이학박사 학위논문

Lsd1 및 *Neurl 1, Neurl 2* 형질전환 생쥐를 이용한 번역 후 변형이 학습과 기억의 조절에 미치는 영향에 대한 연구

Studies on the effect of post-translational modification on learning and memory using transgenic mice of *Lsd1* and *Neurl 1/2*

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뇌과학협동과정

이 재 현

ABSTRACT

Studies on the effect of post-translational modification on learning and memory using transgenic mice of *Lsd1* and *Neurl 1/2*

Jaehyun Lee

College of Natural Science

Interdisciplinary Program in Neuroscience,

Seoul National University

Numerous molecular signaling pathways are engaged in the regulation of learning and memory. A growing number of reports provide that post-translational modification is important for learning and memory. Nonetheless, there is still a lot to be discovered. In this thesis, among the various post-translational modification mechanisms, I focused on the role of phosphorylation and ubiquitination in the regulation of learning and memory. To do this, I used two strains of transgenic mice. The first strain of mice was PKCα-mediated phosphorylation-defective Lysine-specific demethylase 1 (*Lsd1*) knock-in mice. The second strain of mice consisted of

three types of transgenic mice wherein *Neurl 1* gene (*Neurl 1* KO) or *Neurl 2* gene (*Neurl 2* KO) was knocked-out, or *Neurl 1* and *Neurl 2* gene were both knocked-out (*Neurl* 1,2 KO).

In the first part of the current study, I have identified the function of the phosphorylation of Lsd1, mediated by PKCα, in learning and memory. *Lsd1* KI mice showed impaired hippocampus-dependent fear and spatial memory. In addition, *Lsd1* KI mice showed altered presynaptic function and short-term synaptic plasticity; however, long-term synaptic plasticity, such as long- term potentiation (LTP) and long-term depression (LTD), was intact. Consistent with this, RNA-seq analysis of the hippocampus of *Lsd1* KI mice provided that the gene expressions related to presynaptic function-related genes were altered. These results suggest that PKCα-mediated phosphorylation of Lsd1 is involved in the regulation of short-term synaptic plasticity and hippocampus-dependent memory.

In the second part of the study, I have elucidated the specific functions of Neurl 1 and Neurl 2, which are both E3 ubiquitin ligase enzymes in hippocampus-dependent learning and memory. In sum, the results showed that hippocampus-dependent spatial learning and memory were impaired only in *Neurl 1,2* KO mice. In addition, protein synthesis-dependent LTP was impaired only in *Neurl 1,2* KO mice, nonetheless basal synaptic properties have not been altered. Moreover, I revealed that there was neither compensatory overexpression of *Neurl 1* transcripts in *Neurl 2* KO mice nor that of *Neurl 2* transcripts in *Neurl 1* KO mice. Therefore, these findings suggest that hippocampus-dependent spatial memory and protein-synthesis dependent LTP were impaired when *Neurl 1* and *Neurl 2* are both absent, but not

when either *Neurl 1* or *Neurl 2* is present.

Taken together, I have identified two cases in which post-translational modification is involved in the regulation of learning and memory, one concerning the effect of PKCα mediated-phosphorylation of Lsd1, and the other about the role of E3 ubiquitin ligases, Neurl 1 and Neurl 2. Even though it is hard to say that *Lsd1* and *Neurl 1* and *Neurl 2* gene, per se, share the same molecular pathway in regulating learning and memory, these studies suggest that the phosphorylation of Lsd1 and the expression of either *neurl 1* or *neurl 2* is essential, in regulating hippocampus-dependent spatial learning and memory. Thus, this thesis provides multiple pieces of evidence for the fact that post-translational modification provides multiple conduits through which regulation of learning and memory could be achieved.

Keywords: Phosphorylation, Ubiquitination, Synaptic plasticity, Histone demethylase, E3 ligase, Hippocampus

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

BACKGROUND

Post-translational modification

Post-translational modification is a critical biochemical process involved in the diversification of protein functions and the regulation of various cellular events such as gene expression, protein-protein interaction, and cellular signal transduction (Routtenberg and Rekart 2005, Walsh 2006, Sunyer, Diao et al. 2008, Deribe, Pawson et al. 2010, Nussinov, Tsai et al. 2012, Hasegawa, Yoshida et al. 2014, Lussier, Sanz-Clemente et al. 2015). There exist many different types of post-translational modifications, such as acetylation, methylation, phosphorylation, ubiquitination, and SUMOylation. Each process modulates the structural and functional changes of proteins through enzymatic modification which add functional groups to target substrates following protein biosynthesis. For instance, Acetylation/deacetylation and methylation/demethylation are well studies post-translational modification in epigenetic regulation of gene expression through histone modification (Bannister and Kouzarides 2011). Protein kinases/phosphatases regulate activity of target substrates such as receptors and enzymes through attachment or detachment of phosphate (Ardito, Giuliani et al. 2017). Ubiquitination involves an enzymatic cascade which leads to the degradation of target substrates by means of ubiquitin-proteasome system (Nandi, Tahiliani et al. 2006). SUMOylation is mediated by the function of Small Ubiquitin-like Modifier (SUMO) proteins in gene transcription, cell cycle, and subcellular transport (Hay 2005). These multifaceted pieces of evidence testify the fact that post-translational modification plays a role in various biological functions.

Protein phosphorylation involved in the regulation of learning & memory and synaptic plasticity

Phosphorylation is an enzymatic reaction in which a phosphate group is added to target proteins. Phosphate groups primarily attach to serine, threonine, or tyrosine residues (Brady and Siegel 2012). Two kinds of enzymes regulate the phosphorylation of protein: protein kinases and protein phosphatases. Protein kinases phosphorylate specific target proteins, while protein phosphatases remove amino acid residue of its substrate proteins (Manning, Whyte et al. 2002).

Accumulating evidence suggests that protein kinases and protein phosphatases are also engaged in synaptic plasticity, especially in long-term potentiation (LTP) (Pasinelli, Ramakers et al. 1995) and long-term depression (LTD) (Lee 2006). Involvement of protein kinase M ζ (PKMζ) (Sacktor, Osten et al. 1993, Serrano, Yao et al. 2005), Ca2+/calmodulin-dependent protein kinase II (CaMKII) (Silva, Stevens et al. 1992, Giese, Fedorov et al. 1998), cAMP-dependent protein kinase (PKA) (Matthies and Reymann 1993, Abel, Nguyen et al. 1997) and various kinases are required for the regulation of LTP. Moreover, PKA (Brandon, Zhuo et al. 1995, Kameyama, Lee et al. 1998) and several protein phosphatases such as protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A) and protein phosphatase 2B (PP2B, calcineurin) (Mulkey, Herron et al. 1993, Mulkey, Endo et al. 1994) are involved in the regulation of LTD.

Furthermore, previous studies using transgenic mice in which phosphorylation deficit occurred to M3-muscarinic receptor (Poulin, Butcher et al. 2010), TrkB (Lai,

Wong et al. 2012), and AMPA Receptor GluR1 Subunit (Lee, Takamiya et al. 2003) showed impairments in spatial memory and synaptic plasticity.

Protein ubiquitination involved in the regulation of learning & memory and synaptic plasticity

Ubiquitin is a 76–amino acid polypeptide, which can be covalently attached to lysine residues in substrate proteins (Hershko and Ciechanover 1998). Ubiquitination is a process in which target substrates are modified through an enzymatic cascade comprising ubiquitin-activating enzymes (E1 ligase), ubiquitin-conjugating enzymes (E2 ligase), and ubiquitin ligases (E3 ligase) (Wilkinson 1987, Song and Luo 2019). In brief, activated E1 ligase first activates ubiquitin; then, activated ubiquitin is transferred and conjugated to E2 ligase; depending on the E3 ubiquitin ligase, E2-ubiquitin conjugate can be transferred to the protein substrate (Hershko and Ciechanover 1998). This modification induces a change in properties of substrate proteins, including protein activity, intracellular trafficking, cellular localization, protein-protein interaction, and proteasomal degradation (Hicke 2001).

Ubiquitination plays an important role in modulating overall synaptic plasticity, including synapse formation, elimination, LTP, and LTD (Haas and Broadie 2008, Mabb and Ehlers 2010). In specific, ubiquitination is reported to be crucial in a number of processes which enable some of the functionalities related to neuronal receptors known to regulate synaptic plasticity, such as AMPA receptors trafficking (Widagdo, Guntupalli et al. 2017), activity-dependent degradation of NMDA receptors (Kato, Rouach et al. 2005), and regulation of kainate receptors (KARs) and metabotropic glutamate receptors (Lin and Man 2013).

In addition, previous reports suggested diverse roles of various E3 ligases in synaptic plasticity, learning and memory (Zhang, Li et al. 2013, Chakraborty, Paul et al. 2015, Kim, Kim et al. 2015, Sun, Zhu et al. 2015). For instance, transgenic mice lacking a type of ubiquitin E3, *UBE3A*, in the brain exhibited impaired contextual fear learning and LTP (Jiang, Armstrong et al. 1998). Another study that used a knock-out transgenic mice of an E3 ligase, *Dorfin,* showed impaired contextual fear memory, but not in other kinds of memories, and enhanced LTP (Park, Yang et al. 2015).

Studies of hippocampus-dependent memory in rodent model

Over the past decades, numerous lines of transgenic mice were produced and this provided a novel opportunity for approaching specific biological functions in those model mice. Even more, cognitive functions, such as learning and memory, have been accessed using rodent models.

Spatial memory is a well-studied form of memory in rodent models. Researchers assess spatial memory using various behavioral paradigms such as Barnes maze test (Barnes 1979), object location memory test (Murai, Okuda et al. 2007, Vogel-Ciernia and Wood 2014), and Morris water maze test (Brandeis, Brandys et al. 1989, Vorhees and Williams 2006). Among these tests, the Morris water maze test is considered as a test for hippocampus-dependent spatial memory (Morris, Garrud et al. 1982), because this test requires mice to memorize and utilize spatial cues, such as the location of objects within the surrounding, in order to reach a platform concealed under opaque water.

Fear memory is one of the best-studied memory in rodent models. Previous fear memory studies about contextual fear learning and cued fear learning provided us with

a deeper understanding of fear memory (Wehner and Radcliffe 2004). The contextual fear conditioning test is one of the most popular behavioral tasks for testing hippocampus-dependent forms of memory (Fanselow 2000). Since fear learning is a sort of Pavlovian conditioning (Maren and Holt 2000), association between the context (CS) and foot shock (US) is formed during the experimental paradigm of contextual fear conditioning.

LTP and LTD in the hippocampus

Synapses are modified in an activity-dependent way. On the one hand, persistent stimulation induces a strengthened connection between a pre- and postsynaptic terminal, in a process termed LTP. On the other hand, unpaired activation of pre- and postsynaptic terminals induces long-lasting depression, or LTD. Researches on LTP and LTD have provided a much deeper understanding regarding the molecular mechanisms of synaptic plasticity (Bear and Malenka 1994). In this study, I investigated two forms of LTP: early-phase LTP (E-LTP) and late-phase LTP (L-LTP). E-LTP requires a signal transduction cascade by several kinases that phosphorylate essential molecules, including ion channels in neurons. However, the effects of E-LTP ebbs away within several hours. In contrast, while L-LTP requires *de novo* protein synthesis, it continues to exist for several hours in vitro and persists for weeks or even months in vivo (Santini, Huynh et al. 2014). Moreover, LTP and LTD are also important for synaptic plasticity in learning and memory (Collingridge, Peineau et al. 2010, Nicoll 2017).

PURPOSE OF THIS STUDY

Among multiple types of post-translational modifications, phosphorylation and ubiquitination are reversible and have been reported to play crucial roles in learning and memory and synaptic plasticity. However, there still remains a lot to be revealed. Here, I have focused on the effect of phosphorylation and ubiquitination on learning and memory in terms of behavior, physiological and molecular mechanisms.

In chapter II, I examine specific effects of phosphorylation of Lsd1 mediated by PKCα on hippocampus-dependent learning and memory. Using PKCα-mediated phosphorylation-defective *Lsd1* KI mice, I conduct behavioral and physiological tests for learning and memory. First, I demonstrate the hippocampus-dependent learning and memory and physiological property of *Lsd1* KI mice. Second, I introduce phosphorylation-defective Lsd1 induced alterations of gene expression. I sort out changes in mRNA expression levels using RNA-seq and qRT-PCR.

In chapter III, I identify specific functions of Neurl 1 and Neurl 2, which are E3 ligase enzymes, in hippocampus-dependent learning and memory. Using single knock-out mice of *Neurl 1* or *Neurl 2*, and knock-out mice of both *Neurl 1* and *Neurl 2*, I performed behavioral and physiological tests for learning and memory. First goal of this study is to underline the role of Neurl 1 and Neurl 2 in hippocampus-dependent learning and memory. Second goal of this study is to check if there occurred any alteration in the expression levels of downstream molecules of *Neurl 1* and *Neurl 2* and if there existed compensatory mechanisms between the two genes for making amends for the loss of one of the genes.

CHAPTER II

PKCα-mediated phosphorylation of Lsd1 is required in presynaptic plasticity and hippocampus-dependent learning and memory

INTRODUCTION

Lysine-specific demethylase 1 (Lsd1), also referred to as KDM1, is a histone-specific demethylase. Lsd1 acts on mono-and di-methylated histone H3K4 or H3K9 via amine oxidation reaction which requires flavin adenine dinucleotide (FAD) (Shi, Lan et al. 2004, Yang, Gocke et al. 2006). Lsd1 form CoREST complexes together with histone deacetylase (HDAC) 1 and 2 which contribute in the repression of certain genes and interact with androgen receptor (AR) to induce the activation of AR-dependent genes (Metzger, Wissmann et al. 2005, Wang, Hevi et al. 2009, Rudolph, Beuch et al. 2013). On the other hand, Lsd1 also plays an important role in embryogenesis, tissue differentiation process, and tumor cell growth (Kahl, Gullotti et al. 2006, Lim, Janzer et al. 2010, Pedersen and Helin 2010). Moreover, Lsd1 represses Notch signaling by forming a SIRT-LSD1 co-repressor complex (Mulligan, Yang et al. 2011).

Previous studies reported that Lsd1 plays a role in learning and memory. In the novel object recognition (NOR) task, inhibition of Lsd1 by treatment of a specific inhibitor, RN-1, immediately following a novel object recognition training resulted in a long-term memory deficit in the NOR task. However, short-term memory of the subject mice was intact (Neelamegam, Ricq et al. 2012). Lsd1-mediated histone lysine methylation on H3K9 results in gene expressions needed for fear memory consolidation (Gupta, Kim et al. 2010, Gupta-Agarwal, Jarome et al. 2014). In addition, it was reported that an alternatively spliced neuronal isoform of Lsd1 (Lsd1n) is also produced in mammalian (Zibetti, Adamo et al. 2010). Instead of H3K4 demethylase activity, Lsd1n has a demethylase activity upon histone H4K20

and is involved in long-term memory formation via control of transcriptional elongation (Wang, Telese et al. 2015).

Moreover, a recent study reported that phosphorylation of Lsd1 at Serine 112 residue is mediated by PKCα (Nam, Boo et al. 2014). Several studies suggested that PKCα-mediated phosphorylation of Lsd1 is implicated in the induction of epithelial-mesenchymal transition and metastasis of breast cancer (Feng, Xu et al. 2016), regulation of inflammatory response (Kim, Nam et al. 2018) and circadian rhythmicity (Nam, Boo et al. 2014). However, the function of the phosphorylation of Lsd1 by PKCα in cognitive capabilities still awaits to be elucidated. To shed light on this issue, I investigated how phosphorylation of Lsd1 by PKCα affects the regulation of both learning and memory and synaptic plasticity.

In this study, I used transgenic mice expressing PKCα mediated phosphorylation-defective *Lsd1*, henceforth referred as *Lsd1* KI mice. My results provide that *Lsd1* KI mice show altered presynaptic plasticity and impaired hippocampus-dependent learning and memory. In addition, I revealed that the expression levels of memory and presynaptic function-related genes were altered in the hippocampus of *Lsd1* KI mice.

EXPERIMENTAL PROCEDURES

Mice

Production of a defective form of *Lsd1* knock-in (*Lsd1* KI) mice was conducted according to a previously established protocol (Nam, Boo et al. 2014). As a brief explanation, PKCα phosphorylates the 112th serine residue of Lsd1. However, *Lsd1* KI mice express phosphorylation-defective Lsd1, in which serine 112 is replaced into alanine. Mice were co-housed and provided with food and water ad libitum. The animals were subjected to 12-hour dark/light cycle (lights on at 9:00 a.m., lights off at 9:00 p.m.). Adult male mice between age of 8-15 week were used, and behavioral experiments were performed during the light phase. All tests were performed as blind tests with regard to the information of genotypes. This research was endorsed by Seoul National University's Institutional Animal Care and Use Committee. All experiments were conducted in compliance with the institution's tabulated guidelines and regulations.

Immunohistochemistry

Sample preparation

Cardiac perfusion was performed using a 4% solution of paraformaldehyde (PFA) dissolved in 1x PBS. Brains extracted from *Lsd1* KI mice and WT littermates were stored in the 4% PFA solution overnight at 4 ° C. Using a cryostat machine, the brain was sectioned into 40 μm-thick slices.

Antibody staining & Imaging

Brain sections were incubated in 2% goat serum blocking solution (0.2% Triton X-100 in PBS) for 1 hour. After the first blocking step, Lsd1 antibodies (1:500, Abcam), dissolved in a blocking solution with the same composition, were applied to the brain sections at 4 °C overnight. The next day, anti-rabbit Alexa Fluor 555 IgG (1:400, Invitrogen) in the blocking solution was treated to the brain sections and the sections were incubated at room temperature for 2 hours. A fluorescent microscope (IX51, Olympus) was employed while capturing images with fluorescent signals.

Behavioral tests

Open-field test

A white plexiglas box (40 × 40 × 40 cm) was used as an open field box. Mice were put into an empty open field box and were permitted to explore freely in a dim light condition. Time spent in each of two zones (Center zone (within a 20 × 20 cm) and the peripheral zone (40 × 40 cm)) and the total moved distance were calculated using a tracking program (EthoVision 9.0, Noldus).

Elevated zero maze test

The elevated zero maze (EZM) apparatus used in this study was a round track (60 cm diameter, 5 cm width) elevated 65 cm above ground level. EZM apparatus consisted of four zones: two zones had walls on both sides (closed arms) and the

other two were without walls (open arms). Mice were positioned on one of the closed arms and freely explored the apparatus for 5 minutes. The movement of mice in each arm was quantified via a tracking program (EthoVision 9.0, Noldus). Increased time spent in closed arms was considered as an indicator of high-level basal anxiety.

T-maze test

Mice were group-housed and fed 80-85% of the average daily consumption as enforcement of dietary restriction. The T-maze apparatus was made of black walls and white floor made out of acrylic (long arm = 41 cm × 9 cm × 10 cm, short arms = 30 cm × 9 cm × 10 cm, start box = 8 cm × 8 cm × 10 cm). For three consecutive days, mice were handled by the experimenter for 3 minutes a day. Habituation sessions were performed on two consecutive days. During the habituation sessions, 50% condensed milk reward (diluted with saline) was given at the end of the two long arms. Mice were allowed to move freely in the T-maze for 15 minutes. Tests were conducted for five consecutive days from the day after the habituation period, and mice were tested four times a day. All tests are performed under dim light, and each trial consisted of a forced run and a choice run. The forced run arm and the choice run arm were switched in each successive trial as a measure of counterbalancing.

Contextual fear conditioning test

For three consecutive days, the experimenter handled mice for 3 minutes per day. For conditioning, mice were put into a chamber and allowed to explore it freely. Then a foot shock (Single shock, 0.6 mA for 2 sec) was presented through the floor

grid. After the conditioning, the mice were returned to their respective home cages. After the conditioning, the mice were re-exposed to the same chamber for 3 min at either 1 hour or 24 hours after training. The state of immobility, excluding respiration, was regarded as a manifestation of freezing behavior. The level of freezing was automatically evaluated using computer software (Freeze Frame, Coulbourn Instruments).

Cued fear conditioning test

The experimenter handled mice for 3 minutes each for four consecutive days. On the day of the conditioning, the mice were placed in a conditioning chamber, and a 30-second tone (3 kHz, 80 dB) was delivered twice (at 3 min and 5 min). When the tone was finished, an electrical foot shock was immediately released (0.7 mA for 2 sec). One day after the conditioning, subject mice were introduced into a different chamber, which was considered as a distinctive context, for 3 minutes and the same tone was played for another 1 minute. The percentage of freezing behavior was automatically calculated using a computer program (Freeze Frame, Coulbourn Instruments).

Morris water maze test

Mice were handled 3 min per day for three consecutive days prior to the training. For training, mice were put into a round tank (140 cm diameter, 100 cm height), filled with opaque white water (20~22 °C), situated in a room with multiple spatial cues on the walls. I divided the tank into four virtual quadrants, and a platform (10 cm diameter) was positioned at the center of the target quadrant (TQ). During

training days, the experimenter observed whether the subject mouse reached the platform successfully and stayed more than a second on the platform, and rescued the mouse 10 seconds after the observation. Four trials per day were conducted, and 2 min interval was given between trials. Mice were randomly loosed from the edge of the maze and trained to attain the platform within a 60-second-period. When the mouse failed to reach the platform within 60 seconds, they were guided and positioned for 10 seconds on the platform. In probe tests, mice were allowed to explore the tank without the platform; the movement of mice was tracked for 1 minute. Probe tests were conducted twice: on day 4 before starting the training session for that day, and on day 6. A tracking program (EthoVision 9.0, Noldus) was used for analysis.

Three-chamber test

The procedure of the three-chamber test was conducted over five consecutive days and was composed of two parts: four days of handling period and one test day. During the first four days, the experimenter handled stranger mice, with which the test mice never acquainted, for 3 minutes and then habituated them in a wired cage located in the three-chamber apparatus for 5–10 minutes. On the fourth day, after the end of habituation for a stranger mouse, another mouse (the test mouse) was introduced to the three-chamber apparatus and habituated for 10 minutes. On the fifth day, a sociability test and a social recognition test were serially performed. First, the test mouse was brought to the middle chamber while the doors opening to the other areas were shut. In the other two chambers, two wired cages were positioned. Cage on one side contained a same-sex mouse (stranger 1), while cage on the other

side was empty. Then the doors were opened and the test mouse was allowed to approach the two wired cages. 10 minutes later, the test mouse was put into the middle chamber again, and the doors were closed. For the social recognition test, an empty wire cage was removed, and another same-sex mouse (stranger 2) in a wired cage was situated instead. The doors were again opened, and the test mouse was permitted to access two wired cages. For each set of tests, positions for the two wired cages, one with strangers 1 and one empty (or with stranger 2), were counterbalanced. During the two tests, movement of mice was tracked by tracking software (EthoVision 9.0, Noldus).

Electrophysiology

Extracellular field recordings

Transverse hippocampal slices (thickness 400 μm) were prepared from 4~5-week-old mice (for LTD) and 8~12-week-old mice (for LTP) for extracellular field recordings. The brain tissues extracted from deeply anesthetized mice (isoflurane anesthetization) were sectioned by a manual tissue chopper. Brain slices were allowed to recover for 2 hours and then placed in a recording chamber at 25 °C, perfused (1~1.5 mL/min) with oxygenated artificial cerebrospinal fluid (ACSF, 290 Osm) containing (in mM) 124 NaCl, 2.5 KCl, 1 NaH2PO4, 25 NaHCO3, 10 glucose, 2 CaCl2, and 2 MgSO4. Extracellular field EPSPs (fEPSPs) were recorded in the CA1 region of hippocampal slices using a glass electrode filled with ACSF (1 MΩ). Using concentric bipolar electrodes (MCE-100; Kopf Instruments), the Schaffer collateral (SC) pathway was stimulated every 30 seconds. For measurement, field

potentials were amplified, low-pass filtered (GeneClamp 500; Axon Instruments), and digitized (NI PCI-6221; National Instruments). Using the WinLTP program, data were monitored, analyzed online, and then reanalyzed offline. After a stable baseline was recorded, LTP was induced by high-frequency stimulation (100 Hz, 1 s for HFS-LTP), and four trains of high-frequency stimulation (4 × 100 Hz, 1 s each, 5 minutes inter train interval for HFS-L-LTP). After a stable baseline was recorded, LTD was induced by low-frequency stimulation (1 Hz, 900 stimuli for LFS-LTD), theta-burst stimulation (3 × TBS, 1 s each for TBS-LTP), or (R, S)-3,5-Dihydroxyphenylglycine (DHPG) (100 μM for 10 minutes for DHPG-LTD).

Whole-cell patch-clamp recordings

A vibratome (VT1200S; Leica) was used to prepare 300 μm hippocampal slices and to incubate these slices in a recovery chamber for at least 1 hour. After recovery, the CA3 region was incisioned in the slice, and then the hippocampus tissue was moved to a recording chamber to maintain the RT with oxygenated ACSF. In the case of experiments for test of miniature excitatory post synapses current (mEPSC), the recording pipettes (3~5 MΩ) were filled with an internal solution containing (in mM) 100 Cs-gluconate, 5 NaCl, 10 HEPES, 10 EGTA, 20 TEA-Cl, 3 QX-314, 4 MgATP, and 0.3 Na3 GTP (280~300 mOsm, pH adjusted to 7.2 with CsOH). For blocking the GABA receptor-mediated current, picrotoxin (100 μM) has been added to the ACSF. Additional tetrodotoxin (1μM) was added for the mEPSC measurement. For the spontaneous inhibitory postsynaptic current (sIPSC) recording, I used the following internal solution (in mM): 145 KCl, 5 NaCl, 10 HEPES, 10 EGTA, 10 QX-314, 4 MgATP, and 0.3 Na3GTP (280~300 mOsm, pH

adjusted to 7.2 with KOH) in the presence of AP5 (50 μM) and CNQX (100 μM). Using a Multiclamp 700B (Molecular Devices), the hippocampal neurons were voltage-clamped at −70 mV. The analysis included only cells with a change in access resistance of < 20%. For mEPSC and sIPSC analyses, I used the MiniAnalysis program (Synaptosoft).

RNA-seq analysis

By using TRIzol reagent (Invitrogen), total cellular RNA was extracted from hippocampal tissues of *Lsd1* KI mice and WT littermates. BioAnalyzer tested the purity of extracted RNA. In preparing libraries for RNA-Seq, the standard Illumina protocol was imposed. DNA fragments in libraries having insertion sizes of 300 bp or less were isolated and amplified by using a gel electrophoresis method. Then, using an Illumina HiSeq 2000 sequencer, The DNA fragments in libraries were sequenced in the paired-end sequencing mode (2 × 151 bp reads). To align total sequenced raw reads onto the mouse genome reference sequence (mm10), the GSNAP alignment tool (2013–11–27) [PMID: 20147302] was used. For further analysis, only appropriately and uniquely mapped reading pairs were added. The EdgeR kit [PMID: 19910308] was used to classify the genes that were expressed differently between the *Lsd1* KI mice and WT littermates. Differentially expressed genes were defined in this experiment as the genes that changed their expression level at a minimum of 1.5-fold between samples and 10% cutoff at a false discovery rate (FDR) was further imposed as a criterion on the basis of p values modified by edgeR.

Quantitative real-time PCR (qRT-PCR)

I used TRIzol (Invitrogen) to extract total cellular RNA and then performed reverse transcription with oligo (dT) primers and M-MLV Reverse Transcriptase (Enzynomics). The acquired cDNA was mixed with gene-specific primers and TOPrealTM qPCR 2X PreMIX (SYBR Green, Enzynomics) for qRT-PCR. The quantity of mRNA was detected by using CFX384 TouchTM Real-Time PCR Detection System (Bio-Rad) or ABI 7500 System with SYBR Green. The qRT-PCR cycling conditions were: holding on 95 °C for 15 minutes, followed by 40 cycles of 95 °C for 10 seconds, 60 °C for 15 seconds, and 72 °C for 30 seconds. Amplification of β-actin was used as an internal control and carried out in tandem with each sample.

Table 1. Primer list for qRT-PCR using *Lsd1* KI mice and WT littermates

Crhr1	Forward	CAGCCGCCTACAACTACTTCC
	Reverse	GGTGGAGTACGTGAGTACGATG
Hrh1	Forward	TGTCCGGGTTGCACTTGAA
	Reverse	CTGCCATGATACAACCCAACTG
Hrh3	Forward	GCGTCCCCGACTACTGGTA
	Reverse	GAAGGCTCTACGGAAGCTGTA
Oxtr	Forward	GATCACGCTCGCCGTCTAC
	Reverse	CCGTCTTGAGTCGCAGATTC
Drd2	Forward	GCTCAGGAGCTGGAAATGGA
	Reverse	GGGCTATACCGGGTCCTCTCT
Slc18a2	Forward	CGGCTCCTCTTGCTCATCTG
	Reverse	TGGCGTTACCCCTCTCTTCA
Rab39	Forward	ACTAACCGACGGTCTTTTGAAC
	Reverse	CCAGGTCACATTTATGTCCCAC
Syngr1	Forward	CCCAGGACTACATGGACCC
	Reverse	CAAAGGCGTTGGCTGGATG
Lsd1	Forward	AACTGGCCAAGATCAAGCAAAAA
	Reverse	CAGCTGAATGACAACCTCCAATG
Cplx1	Forward	GAGCGCAAAGCAAAGTACGC
	Reverse	TCTTATACCCTGCCGCATGAC
Ppfibp2	Forward	AGGAAGTCCCGAAGCAAGAGA
	Reverse	CTCCAGTGATTTCTGTGGCAA
Slc32a1	Forward	CGACAAACCCAAGATCACGG
	Reverse	AGGATGGCGTAGGGTAGGC
Vamp1	Forward	CTGGTGGAAAAACTGCAAGATG
	Reverse	CTACCACGATGATGGCACAGA
Bsn	Forward	GCACTCGTACAGCTTAGGCTTTG
	Reverse	GTCCGTGAAGGAGCCATACTG
Ppfia2	Forward	TTAGTGTGGAGCAATGATCGAGTTA
	Reverse	TGCACGCCACTCTCAAGAAT
Rims1	Forward	GGGCAGGATCAGTTCATTTACC
	Reverse	ACTTACACTGGTGCTCTTGATGATG

Statistics

I have conducted either D'Agostino & Pearson omnibus, Shapiro-Wilk or Kolmogorov-Smirnov normality tests to determine whether the data presented here were normally distributed. For analyzing the data from fear conditioning, two-way analysis of variance (ANOVA) was used (between-group factor: genotype; within-group factor: condition). In the analysis of the data from Morris water maze, two-way repeated measure (RM) ANOVA were used for escape latency (between-group factor: genotype; within-group factor: time) and one-way ANOVA analysis was used for quadrant occupancy (% time spent in the quadrant). Also, Bonferroni posttests were performed to evaluate the differences of a pair-wise group. Depending on the result of the normality test, either the Mann Whitney test or unpaired two-tailed t-test was used. The level of significance is indicated as follows: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. GraphPad Prism 5 or 6 program was used. Data were represented as mean ± standard mean error, SEM (or standard deviation, SD).

Data availability

The RNA-Seq data was provided to the NCBI Gene Expression Omnibus (GEO) with an ID of GSE94018.

RESULTS

PKCα-mediated phosphorylation of Lsd1 is required for hippocampus-dependent fear memory

First of all, I examined the gene expression of *Lsd1* in the sub-region of the hippocampus: CA1, CA3, and dentate gyrus. The immunohistochemistry (IHC) experiment was performed using the hippocampus tissues of *Lsd1* KI mice and WT littermates. The signal intensity and localization of Lsd1, detected by Lsd1 antibody, were almost similar in both genotypes (Fig. 1a). These results provide that the phosphorylation deficit of Lsd1 did not affect the expression level of *Lsd1* gene itself.

Next, I conducted contextual fear conditioning (CFC) test to confirm whether the substitution to phosphorylation defective form of Lsd1 leads to a change in hippocampus-dependent fear memory. I tested fear memory at two-time points after training: 1 hour (short-term) and 24 hours (Long-term). Compared to WT littermates, *Lsd1* KI mice showed significantly decreased levels of freezing both in 1 hour (short-term) and 24 hours (Long-term) (Fig. 1b and 1c). These results proposed that short- and long-term contextual fear memory impairment was induced by phosphorylation-defective Lsd1.

To investigate the spatial working memory of *Lsd1* KI mice, I carried out the T-maze test. As a brief reminder, a training trial of the T-maze test consisted of a forced run and a choice run. During the forced run, between two target arms, one of the arms was blocked. Thus, mice mandatorily entered the unblocked arm. During the choice run, however, mice were allowed to choose between two arms. If they choose

to visit the unacquainted arm, they were able to get the reward (50% condensed milk) once more. During the training, WT littermates showed an increase of correct choice, while the correct choice of *Lsd1* KI mice had not increased (Fig. 1d). Consistent with the short-term CFC results, these data showed that phosphorylation-defective Lsd1 induced impairment in formation of spatial working memory.

To investigate whether the effect of phosphorylation-defective Lsd1 was restricted to the hippocampus or extended also to the other brain regions, I performed an amygdala-dependent memory task: auditory fear conditioning (AFC) test. One day after the training, the freezing levels of both genotypes were measured. In contrast with the results of the CFC test, a similar level of freezing was observed in both *Lsd1* KI mice and WT littermates (Fig. 1e). These results indicated that PKCα-mediated phosphorylation of Lsd1 is required for hippocampus-dependent memory but not for amygdala-dependent fear memory.

Figure 1

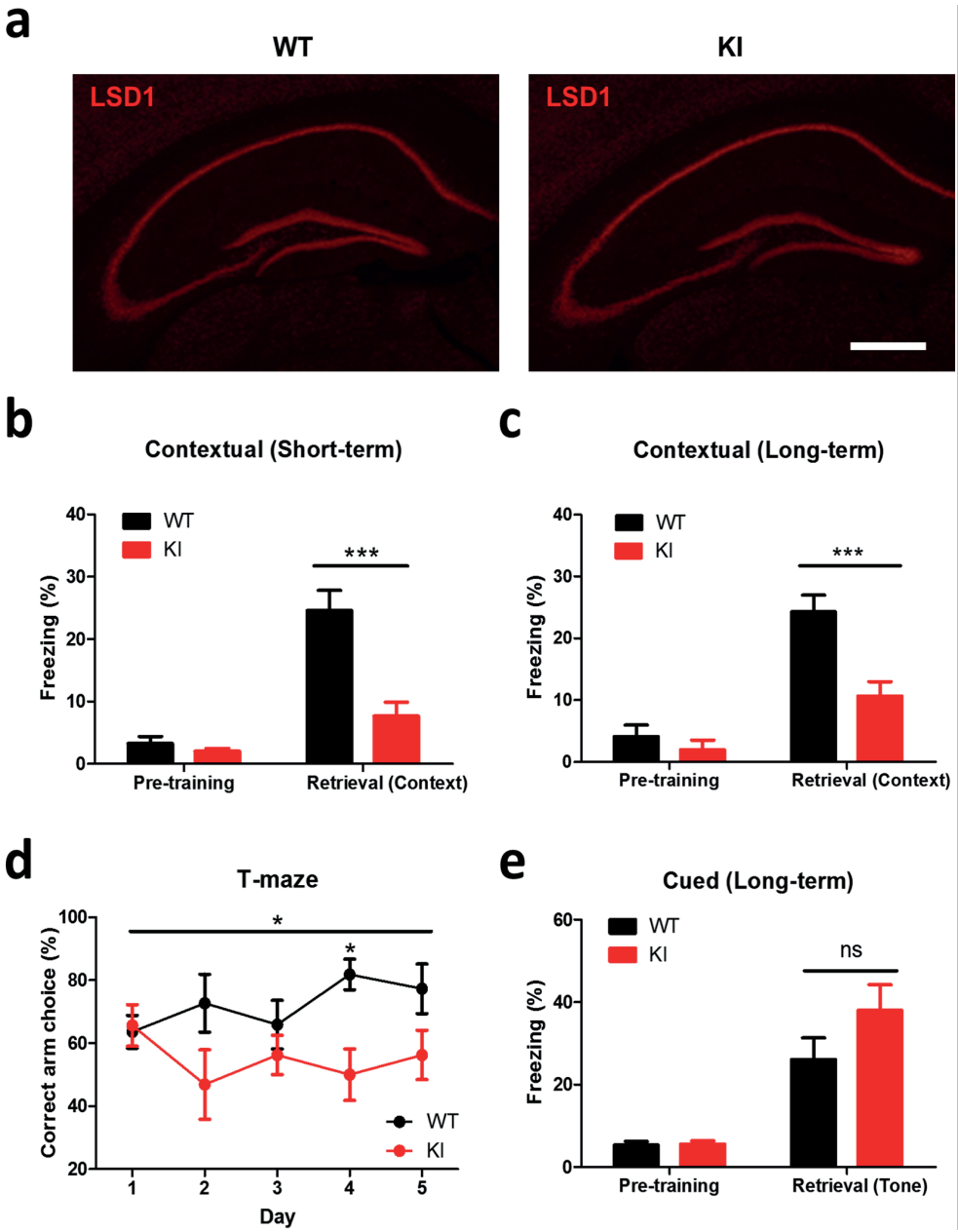
a
WT
KI
LSD1
LSD1
b
Contextual (Short-term)
WT
KI
***
Freezing (%)
40
30
20
10
0
Pre-training
Retrieval (Context)
c
Contextual (Long-term)
WT
KI
***
Freezing (%)
40
30
20
10
0
Pre-training
Retrieval (Context)
d
T-maze
*
*
Correct arm choice (%)
100
80
60
40
20
WT
KI
1
2
3
4
5
Day
e
Cued (Long-term)
WT
KI
ns
Freezing (%)
60
40
20
0
Pre-training
Retrieval (Tone)

Figure 1. PKCα-mediated phosphorylation of Lsd1 is required for hippocampus-dependent fear memory

(a) Representative images of immunohistochemistry. There were no differences in the pattern of Lsd1 expression in the hippocampus of both genotypes. Scale bar: 500 μm (WT: n = 5, KI:n = 5). **(b)** Short-term CFC. During the retrieval, *Lsd1* KI mice (red) exhibited significantly lower level of freezing than WT (black) littermates (WT: n = 7, KI: n = 6; two-way ANOVA, genotype x condition, $F1,22 = 13.58$, $p < 0.01$; effect of genotype, $F1,22 = 18.21$, $p < 0.001$; effect of condition, $F1,22 = 40.07$, $p < 0.0001$; Bonferroni posttests, ***$p < 0.001$). **(c)** Long-term CFC. During the retrieval, *Lsd1* KI mice displayed significantly decreased level of freezing compared to WT (black) littermates (WT: n = 9, KI: n = 9; two-way ANOVA, genotype x condition, $F1,32 = 7.06$, $p < 0.05$, effect of genotype, $F1,32 = 13.36$, $p < 0.001$, effect of condition, $F1,32 = 44.73$, $p < 0.0001$; Bonferroni posttests ***$p < 0.001$). **(d)** T-maze test. The rate of correct arm choice significantly differed between *Lsd1* KI mice and WT littermates. (WT: n = 11; KI: n = 8; two-way RM ANOVA, genotype x time, $F4,68 = 1.83$, $p = 0.133$, effect of genotype, $F1,68 = 7.53$, *$p < 0.05$, effect of time, $F4,68 = 0.37$, $p = 0.826$; Bonferroni posttests, WT vs KI at day 4, *$p < 0.05$). **(e)** AFC. *Lsd1* KI mice displayed a similar level of freezing compared to WT (black) littermates (WT: n = 8, KI: n = 9; two-way ANOVA, genotype x condition, $F1,30 = 1.94$, $p = 0.174$, effect of genotype, $F1,30 = 2.08$, $p = 0.160$, effect of condition, $F1,30 = 40.15$, $p < 0.0001$; Bonferroni posttests, ns: not significant).

PKCα-mediated phosphorylation of Lsd1 is required for hippocampus-dependent spatial memory

To investigate whether phosphorylation defective Lsd1 altered hippocampus-dependent spatial learning and memory, I performed the Morris water maze (WMW) test. I trained both WT littermates and *Lsd1* KI mice for five consecutive days so that the mice can acquire the location of a hidden platform in a round-shape water tank, filled with opaque white water. On day 4 (before training for the day) and day 6, probe tests were performed without the platform for 1 minute (Fig. 2a). In the training sessions, data for escape latencies show that *Lsd1* KI mice spent a significantly longer time to reach the platform compared to WT littermates (Fig. 2b). This data indicates that spatial learning was retarded in *Lsd1* KI mice. During the probe tests, both genotypes were allowed to explore all quadrants in the water tank. In the results of probe test 1, it was observed that WT littermates spent more time in the target quadrant compared to other quadrants, while *Lsd1* KI mice spent nearly equal time in all quadrant (Fig. 2c). This tendency continued in the second probe test, where *Lsd1* KI mice again spent less time in the target quadrant than WT littermates (Fig. 2d). In addition, I compared the mean distance between the subject mouse and the position where the platform was located during the training of both genotypes. *Lsd1* KI mice stayed farther from the location of the platform compared to WT littermates (Fig. 2e). The result demonstrated that PKCα-mediated phosphorylation of Lsd1 is required for hippocampus-dependent spatial memory.

Figure 2

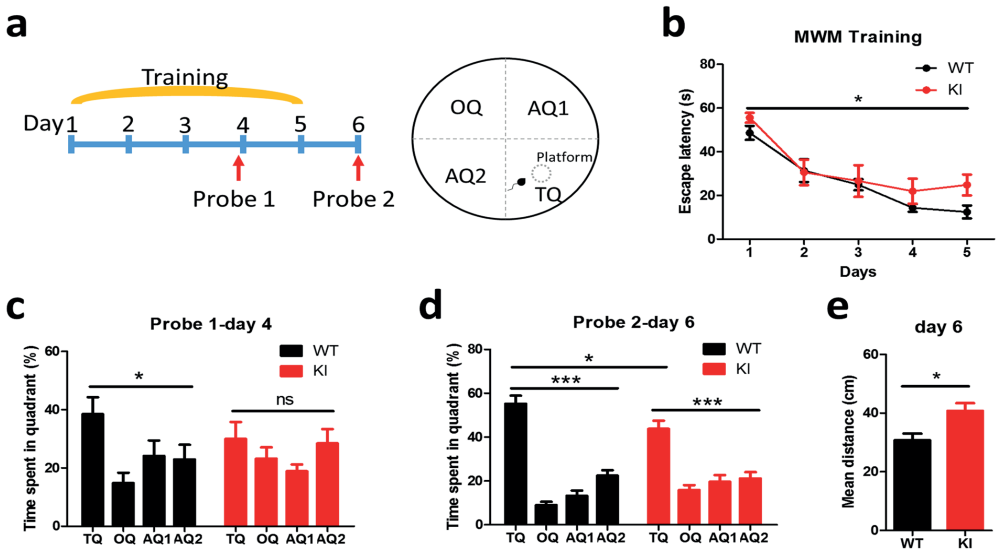
a
Training
Day1 2 3 4 5 6
Probe 1
Probe 2
OQ
AQ1
AQ2
Platform
TQ
b
MWM Training
WT
KI
Escape latency (s)
Days
c
Probe 1-day 4
Time spent in quadrant (%)
ns
TQ OQ AQ1 AQ2 TQ OQ AQ1 AQ2
d
Probe 2-day 6
e
day 6
Mean distance (cm)
WT KI

Figure 2. PKCα-mediated phosphorylation of Lsd1 is required for hippocampus-dependent spatial memory

(a) Timeline of behavior experiment and a mimetic picture of the Morris water maze. **(b)** The learning curve of the MWM task. Escape latency of *Lsd1* KI mice was delayed compared to WT littermates (WT: n = 8, KI: n = 7; two-way ANOVA, genotype x time, $F4,65 = 0.71$, $p = 0.590$, effect of genotype, $F1,65 = 4.03$, $*p < 0.05$, effect of time, $F4,65 = 20.16$, $p < 0.0001$). **(c)** Time spent in each quadrant measured in probe test 1 (day 4). (WT: n = 8, KI: n = 7; one-way ANOVA of WT, $*p < 0.05$, Bonferroni's multiple comparison test, TQ vs OQ, $p < 0.01$; one-way ANOVA of KI, ns $p = 0.307$, Bonferroni's multiple comparison test, TQ vs OQ, ns: not significant). **(d)** Time spent in each quadrant measured in probe test 2 (day 6) (WT: n = 8, KI: n = 7; one-way ANOVA of WT, $***p < 0.0001$; one-way ANOVA of KI, $***p < 0.0001$; unpaired *t*-test, WT vs KI in TQ, $*p < 0.05$). TQ: target, OQ: opposite, AQ1: right, AQ2: left quadrant. **(e)** In probe test 2, mean distance (cm) from the location of the platform was measured (WT: n = 8, KI: n = 7; unpaired *t*-test, $*p < 0.05$).

Lsd1 KI mice show intact basal anxiety and increased locomotion

To investigate the effect of phosphorylation-defective Lsd1 on basal anxiety, I carried out two kinds of anxiety tests: open-field (OF) test and elevated zero maze (EZM) test (Shepherd, Grewal et al. 1994, Prut and Belzung 2003). Mice with a high level of anxiety favor staying near the peripheral zone compared to the center zone in the OF test. Also, mice with a high level of anxiety prefer closed arms compared to open arms in the EZM test. It is because of their nature for avoiding potentially dangerous places. Results of the OF test and EZM test provide that there was no significant difference in anxiety-like behavior between two genotypes (Fig. 3a and 3b). However, *Lsd1* KI mice showed significantly elevated moved distance during the OF test (Fig. 3c). These results suggest that anxiety had not been altered but there rather existed inherent hyperactivity in *Lsd1* KI mice. Thus, these results provide that phosphorylation defective Lsd1 did not affect the mood of the mice.

Figure 3

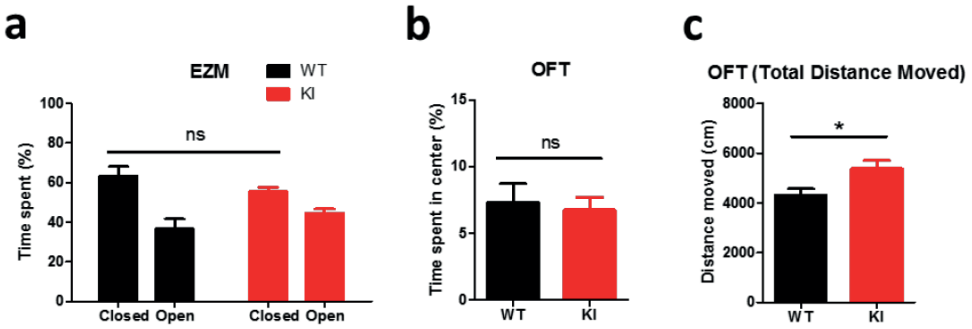

Figure 3. *Lsd1* KI mice exhibit normal basal anxiety and increased locomotion.

(a) EZM test. There were no significant differences in time spent in closed arms between two genotypes (WT: n = 9, KI: n=9; two-way ANOVA, genotype x sector, $F1,32= 4.21$, $p < 0.05$; effect of genotype, $F1,32= 0.00$, $p = 1.000$; effect of sector, $F1,32= 24.57$, $p < 0.0001$; Bonferroni posttests, WT vs KI in closed sector, ns: not significant) **(b-c)** OF test. There were no significant differences in time spent in the center zone between two genotypes (**b,** unpaired *t*-test, ns: not significant), *Lsd1* KI mice showed significantly high level of moved distance compared to WT littermates (**c**, WT: n = 9, *Lsd1* KI: n = 9; unpaired *t*-test, * $p < 0.05$).

Memory for social recognition is impaired in *Lsd1* KI mice

To investigate whether phosphorylation-defective Lsd1 is implicated in sociability and social recognition, I conducted the three-chamber test (Kaidanovich-Beilin, Lipina et al. 2011). In the three-chamber test paradigm, it is regarded that animals with natural sociability prefer to approach a stranger mouse compared to approaching an object. As a result, both genotypes displayed a similar level of social interaction (Fig. 4a and 4b), indicating that sociability was intact in *Lsd1* KI mice. While WT littermates exhibited significantly increased interaction time to a stranger mouse 2 (S2, a novel stranger mouse), interaction times of *Lsd1* KI mice toward stranger mouse 2 and stranger mouse 1 (S1, a familiar mouse) were at comparable levels in the social recognition task (Fig. 4c and 4d). These observations indicate that the lack of phosphorylation of *Lsd1* has affected social recognition memory but not sociability itself.

Figure 4

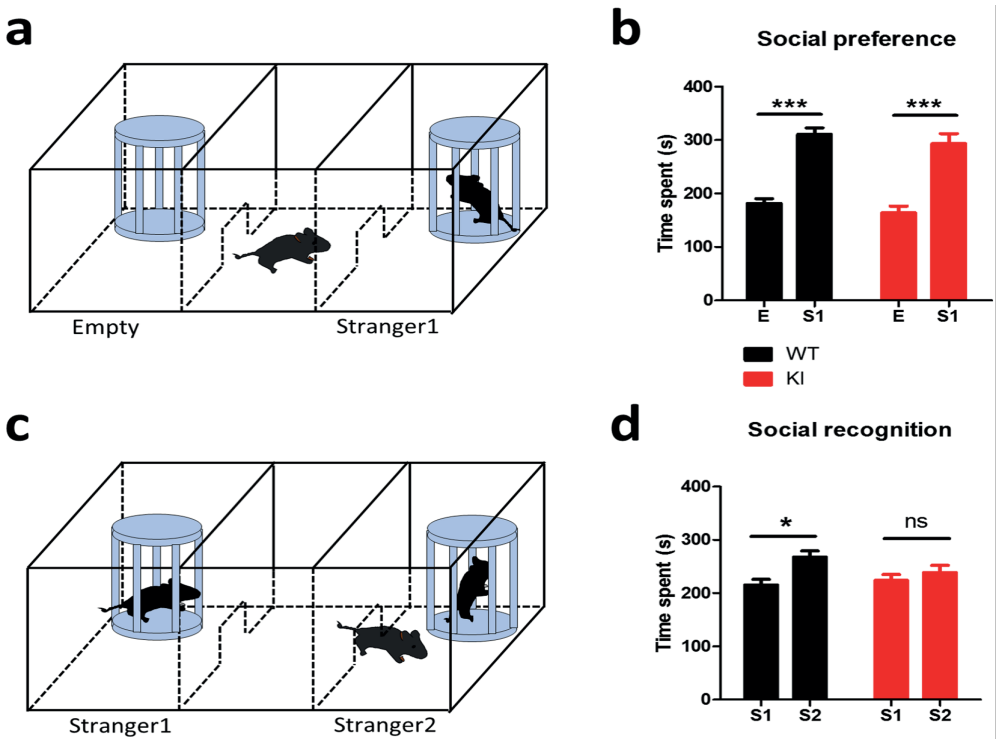
a
Empty
Stranger1
b
Social preference
Time spent (s)
400
300
200
100
0
***
***
E
S1
E
S1
WT
KI
c
Stranger1
Stranger2
d
Social recognition
Time spent (s)
400
300
200
100
0
*
ns
S1
S2
S1
S2

Figure 4. Memory for social recognition is impaired in *Lsd1* KI mice

(a) Mimetic picture of the three-chamber test for social preference test. **(b)** Both *Lsd1* KI mice and WT littermates showed significantly higher exploring time for a stranger mouse (S1) than an empty cup (E) (WT: n = 13, KI: n = 11; two-way ANOVA, genotype x condition, $F1,44 = 0.00$, $p = 1.000$, effect of genotype, $F1,44 = 1.78$, $p = 0.189$, effect of condition, $F1,44 = 58.21$, $p < 0.0001$; Bonferroni posttests, S1 vs E ***$p < 0.0001$). **(c)** Mimetic picture of the three-chamber test for social recognition test. **(d)** *Lsd1* KI mice showed comparable exploring time for the stranger mouse 1 (S1) and the stranger mouse 2 (S2), while WT littermates showed significantly higher exploring time for the stranger mouse 2 (S2) than the stranger mouse 1 (S1) (WT: n = 13, KI: n = 11; two-way ANOVA, genotype x condition, $F1,44 = 2.59$, $p = 0.115$, effect of genotype, $F1,22 = 0.79$, $p = 0.231$; effect of condition, $F1,44 = 8.08$, $p < 0.01$; Bonferroni posttests, S2 vs S1 *$p < 0.05$, ns: not significant). All graphs were plotted as mean ± SEM.

Table 2. Summary of behavioral experiments with *Lsd1* KI mice

Behavior task	*Lsd1* KI
Hippocampus-dependent fear memory (Long-term)	Impaired
Hippocampus-dependent fear memory (Short-term)	Impaired
Amygdala-dependent fear memory (Long-term)	N.S
Hippocampus-dependent Spatial memory	Impaired
Spatial working memory	Impaired
Social preference	N.S
Social recognition	Impaired
Anxiety	N.S

N.S.: Not Significant

Lsd1 KI mice showed changes in presynaptic plasticity

The results of behavioral experiments shown thus far can be summarized as showing the fact that *Lsd1* KI mice exhibit hippocampus-dependent memory impairment. Since synaptic plasticity is supposed to be the underpinning of hippocampus-dependent long-term memory (Bliss and Collingridge 1993, Malenka and Bear 2004), I performed extracellular field recordings to test whether any physiological changes were induced by phosphorylation defective Lsd1. By testing the input-output (I/O) relationship and paired-pulse ratio (PPR), I estimated the basal synaptic transmission of SC-CA1 synapses in *Lsd1* KI mice and WT littermates. Improved I/O relationship (Fig. 5a), and reduced PPR (Fig. 5b) were observed in *Lsd1* KI mice. An increase in the I/O relationship indicates enhanced synaptic transmission, and a decline of PPR suggests increased presynaptic neurotransmitter release probability. Thus, these results provide that the alteration of presynaptic plasticity has occurred by phosphorylation-defective Lsd1.

To earn more specific profiles of this alternation in presynaptic function, I performed the post-tetanic potentiation (PTP) analysis. PTP reflects an improvement of the release of neurotransmitters following high-frequency stimulation on a minute time scale. After high-frequency stimulation, residual Ca2+ ion is accumulated, and this leads to an increase in the release of neurotransmitters triggered by active PKC in the presynaptic terminal (Zucker and Regehr 2002, Fioravante and Regehr 2011). Here, I used the PTP protocol consists of a single train of tetanic stimulation (100 Hz/s) under the presence of D-APV (D(-)-2-amino-5-phosphonovaleric acid) (50 μM) for obstructing the postsynaptic modifications

mediated by NMDA receptor (Lee, Kobayashi et al. 2015, Watabe, Nagase et al. 2016). I observed that PTP was significantly reduced in the *Lsd1* KI mice (Fig. 5c and 5d), indicating that *Lsd1* KI mice have altered presynaptic functions related to short-term synaptic plasticity.

In addition, I conducted whole-cell patch-clamp recording to estimate miniature postsynaptic excitatory current (mEPSC) for basal synaptic transmission. The frequency of mEPSC reflects the presynaptic release of neurotransmitters, and the amplitude of mEPSC indicates the magnitude of postsynaptic potential. *Lsd1* KI mice showed a significantly higher frequency of mEPSC than WT littermates but comparable amplitudes of mEPSC (Fig. 5e and 5f). In line with the results described above, these findings suggested an increased release probability of neurotransmitters in *Lsd1* KI mice.

Figure 5

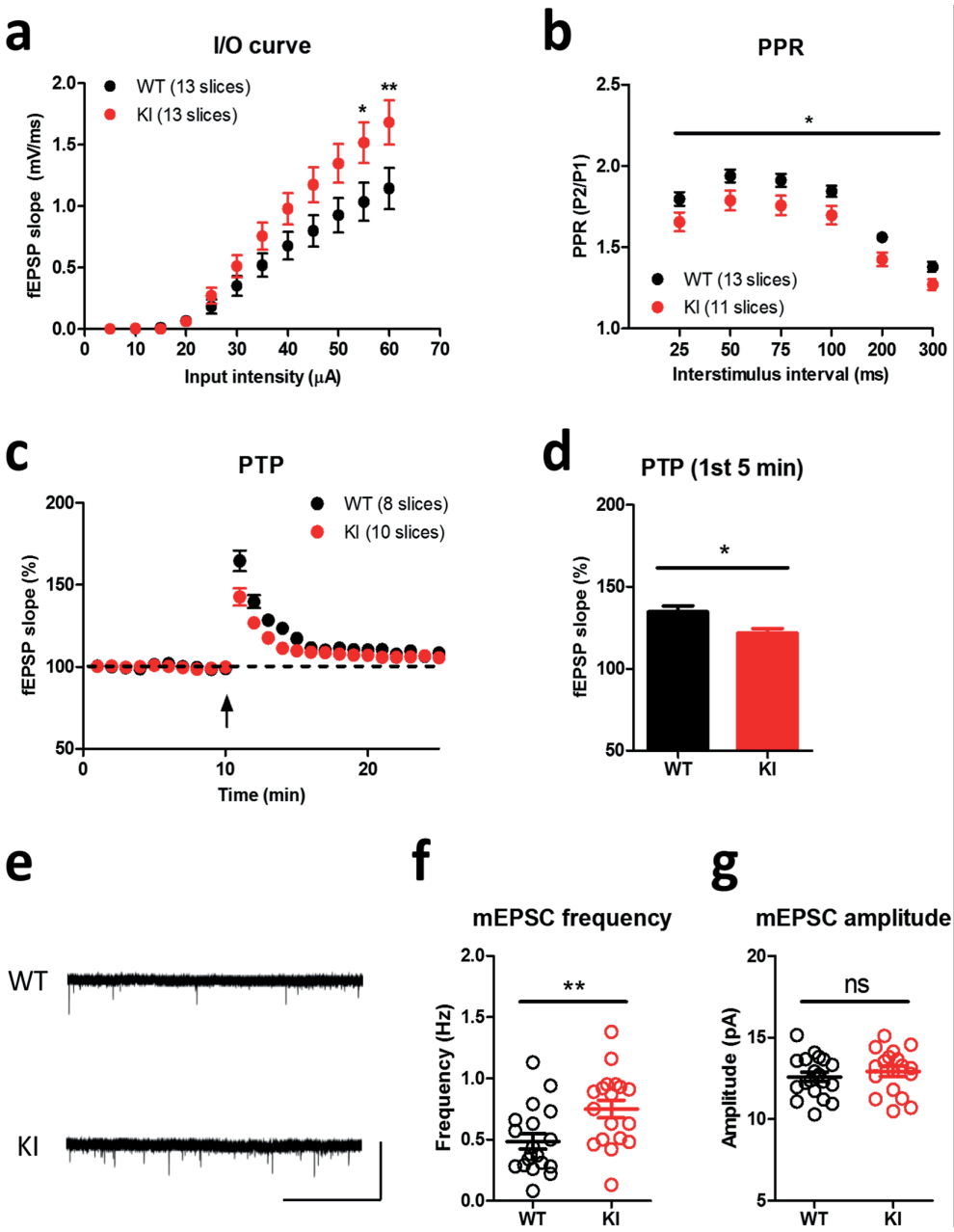
a
I/O curve
WT (13 slices)
KI (13 slices)
fEPSP slope (mV/ms)
Input intensity (μA)
b
PPR
PPR (P2/P1)
WT (13 slices)
KI (11 slices)
Interstimulus interval (ms)
c
PTP
WT (8 slices)
KI (10 slices)
fEPSP slope (%)
Time (min)
d
PTP (1st 5 min)
fEPSP slope (%)
WT
KI
e
WT
KI
f
mEPSC frequency
Frequency (Hz)
WT
KI
g
mEPSC amplitude
Amplitude (pA)
ns
WT
KI

Figure 5. *Lsd1* KI mice showed changes in presynaptic plasticity

(a) The curve of input-output (I/O) relationship at SC-CA1 synapses. *Lsd1* KI mice showed enhanced I/O relations compared to WT littermates (WT: n = 13, KI: n = 13; two-way RM ANOVA, input intensity x genotype, $F11,264 = 4.46$, $p < 0.0001$, effect of input intensity, $F11,264 = 121.54$, $p < 0.0001$, effect of genotype, $F1,264 = 3.42$, $p = 0.077$; Bonferroni posttests $*p < 0.05$, $**p < 0.01$). **(b)** Paired-pulse ratio (PPR) at SC-CA1 synapses. In *Lsd1* KI mice the ratio was significantly decreased in comparison with WT littermates (WT: n = 13, KI: n = 11; two-way RM ANOVA, inter stimulus interval x genotype, $F5,105 = 0.36$, $p = 0.874$, effect of inter stimulus interval, $F5,105 = 245.20$, $p < 0.0001$, effect of genotype, $F1,21 = 6.13$, $*p < 0.05$). **(c)** Post-tetanic potentiation (PTP) at SC-CA1 synapses. *Lsd1* KI mice showed significantly decreased PTP compared to WT littermates (WT, n = 8; KI, n = 10; arrow, 1 × HFS). **(d)** Significant difference observed in PTP for the first 5 minutes of recording between *Lsd1* KI mice and WT littermates (WT: 134.7 ± 3.6%, 8 slices from 5 mice, KI: 121.7 ± 2.8%, 10 slices from 5 mice; unpaired *t*-test, $*p < 0.05$). **(e)** Representative traces of miniature excitatory postsynaptic currents (mEPSCs) recording at SC-CA1 synapses. Scale bar, vertical: 50 pA; horizontal: 10 sec. **(f)** Significantly enhanced frequency of mEPSCs was observed in *Lsd1* KI mice (WT: n = 19, KI: n = 18; unpaired *t*-test, $**p < 0.01$). **(g)** Comparable level of mEPSC amplitudes was observed in both genotypes (WT: n = 19, KI: n = 18; unpaired *t*-test, ns: not significant).

Lsd1 KI mice showed intact long-term synaptic plasticity: LTP and LTD

Next, I checked whether phosphorylation-defective Lsd1 also affected long-term synaptic plasticity. First of all, I examined the E-LTP, induced by high-frequency stimulation (HFS) (Fig. 6a) and theta-burst stimulation (TBS) (Fig. 6b). Contrary to my expectation, there was no significant difference between the genotypes. Moreover, I did not observe any impairment in late-LTP (L-LTP) induced by four pulses of high-frequency tetanus in 5 min intervals (Fig. 6c). Thus, it was observed that phosphorylation-defective Lsd1 affected neither E-LTP nor L-LTP.

Next, I tested the opposite case of the LTP, long-term depression (LTD). I performed two kinds of experiments: NMDA receptor-dependent LTD (Fig. 6d) and mGluR-dependent LTD (Fig. 6e). These two types of LTD were intact in both genotypes. Therefore, these results provide that PKCα mediated phosphorylation of Lsd1 is not involved in the regulation of long-term synaptic plasticity.

Figure 6

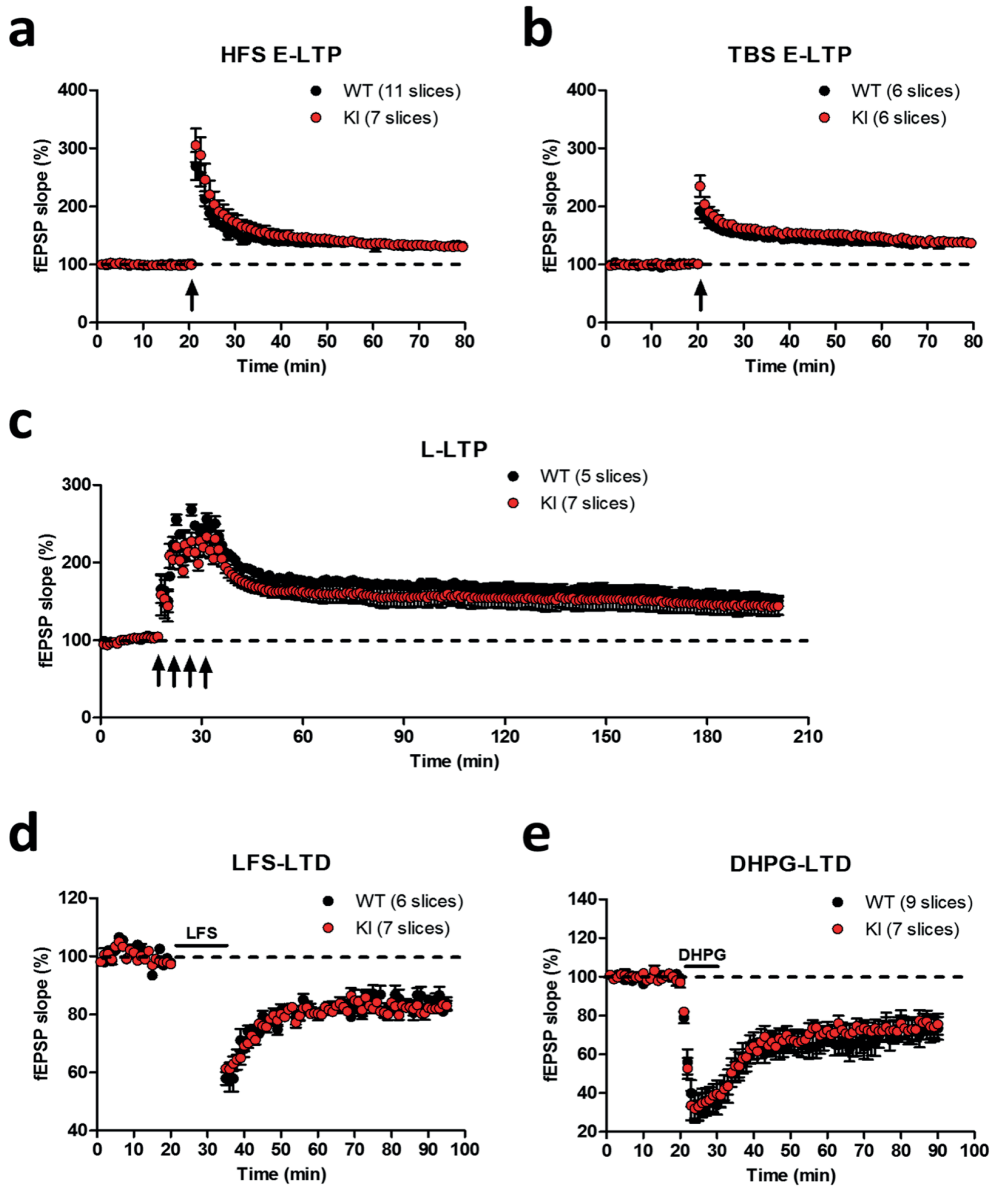
a
HFS E-LTP
WT (11 slices)
KI (7 slices)
fEPSP slope (%)
Time (min)
b
TBS E-LTP
WT (6 slices)
KI (6 slices)
fEPSP slope (%)
Time (min)
c
L-LTP
WT (5 slices)
KI (7 slices)
fEPSP slope (%)
Time (min)
d
LFS-LTD
LFS
WT (6 slices)
KI (7 slices)
fEPSP slope (%)
Time (min)
e
DHPG-LTD
DHPG
WT (9 slices)
KI (7 slices)
fEPSP slope (%)
Time (min)

Figure 6. *Lsd1* KI mice showed intact long-term synaptic plasticity: LTP and LTD

(a) Early LTP (E-LTP) induced by high-frequency stimulation (HFS) at SC-CA1 synapses. There was no difference between the genotypes (WT: n = 11, KI: n = 7). **(b)** TBS E-LTP at SC-CA1 synapses. There was no difference between the two genotypes (WT: n = 6, KI: n = 6). **(c)** HFS L-LTP at SC-CA1 synapses. Both genotypes showed comparable levels of LTP (WT: n = 5, KI: n = 7). **(d)** LFS mediated NMDA-R dependent LTD at SC-CA1 synapses. Both genotypes showed comparable levels of LTP (WT: n = 6, KI: n = 7). **(e)** mGluR-LTD induced by DHPG at SC-CA1 synapses. There is no difference between the genotypes (WT: n = 9, KI: n = 7).

Table 3. Summary of electrophysiological experiments with *Lsd1* KI mice

	Behavior task	*Lsd1* KI
Short-term synaptic plasticity	I/O Curve	Increased
	PPR	Decreased
	PTP	Decreased
	mEPSC frequency	Increased
	mEPSC amplitude	N.S
Long-term synaptic plasticity	E-LTP	N.S
	L-LTD	N.S
	LTD	N.S

N.S.: Not Significant

Distinctive gene expression between *Lsd1 KI* mice and WT littermates

The aforementioned analyses conducted in this research provide that *Lsd1* KI mice exhibit deficits in learning and memory and altered presynaptic plasticity. To achieve an explanation of these phenomena on the level of molecular mechanism, I investigated whether phosphorylation-defective Lsd1 affected the expression of genes related to learning and memory. To do this, mRNA extracted from the hippocampus tissue of *Lsd1* KI mice and WT littermates was used for RNA-seq analysis. By using entire gene expression profiles, I estimated sample distance between the two groups (WT and KI) and replicates (1 and 2). The heat map data showed that the two groups were correctly separated (Fig. 7a). I obtained 271 upregulated, and 110 downregulated genes from 381 differentially expressed genes using differential gene expression analysis (Fig. 7b and 7c). In order to gain an understanding concerning which transcription factors interact with Lsd1 in activating or repressing target genes, I used Enrichr (http:/amp.pharm.mssm.edu/Enrichr/) (Kuleshov, Jones et al. 2016) to examine putative promoters for the differentially expressed genes. Since hippocampus-dependent memory impairment and the altered presynaptic property was observed in *Lsd1* KI mice, I looked into the genes that were expressed differently with an emphasis on presynaptic and postsynaptic plasticity and memory. Postsynaptic function-related genes such as Calcium/calmodulin-dependent protein kinase II (CaMKII; *Camk2a, Camk2b, Camk2d, and Camk2g*), Postsynaptic density protein-95 (PSD-95; *Dlg4*), Shank (*Shank1, Shank2, and Shank3*), SynGAP (*Syngap1*), AMPA-R (*Gria1*), Homer (*Homer1, Homer2 andHomer3*), NMDA-R (*Grin1,*

Grin2a, Grin2b, Grin2c, Grin2d, Grin3a, and Grin3b), Neurolign (*Nlgn1, Nlgn2 andNlgn3*), mGluR (*Grm1, Grm2, Grm3, Grm4 and Grm5*), GKAP (*Dlgap1, Dlgap2, Dlgap3, Dlgap4 and Dlgap5*), Spine-associated RapGAP (SPAR; *Sipa1l1, Sipa1l2, and Sipa1l3*), nNOS (*Nos1, Nos2 and Nos3*), and GRIP (*Grip1*) did not exhibit altered gene expression between *Lsd1* KI mice and WT littermates (Table 4). However, expression of a number of presynaptic function-related genes such as Histamine receptor H1 (*H1R; Hrh1*), Histamine receptor H3 (*H3R; Hrh3*), Dopamine D2 receptor (*D2R; Drd2*), and Vesicular monoamine transporter 2 (*VMAT2; Slc18a2*) were increased in *Lsd1* KI mice (Table 5).

Figure 7

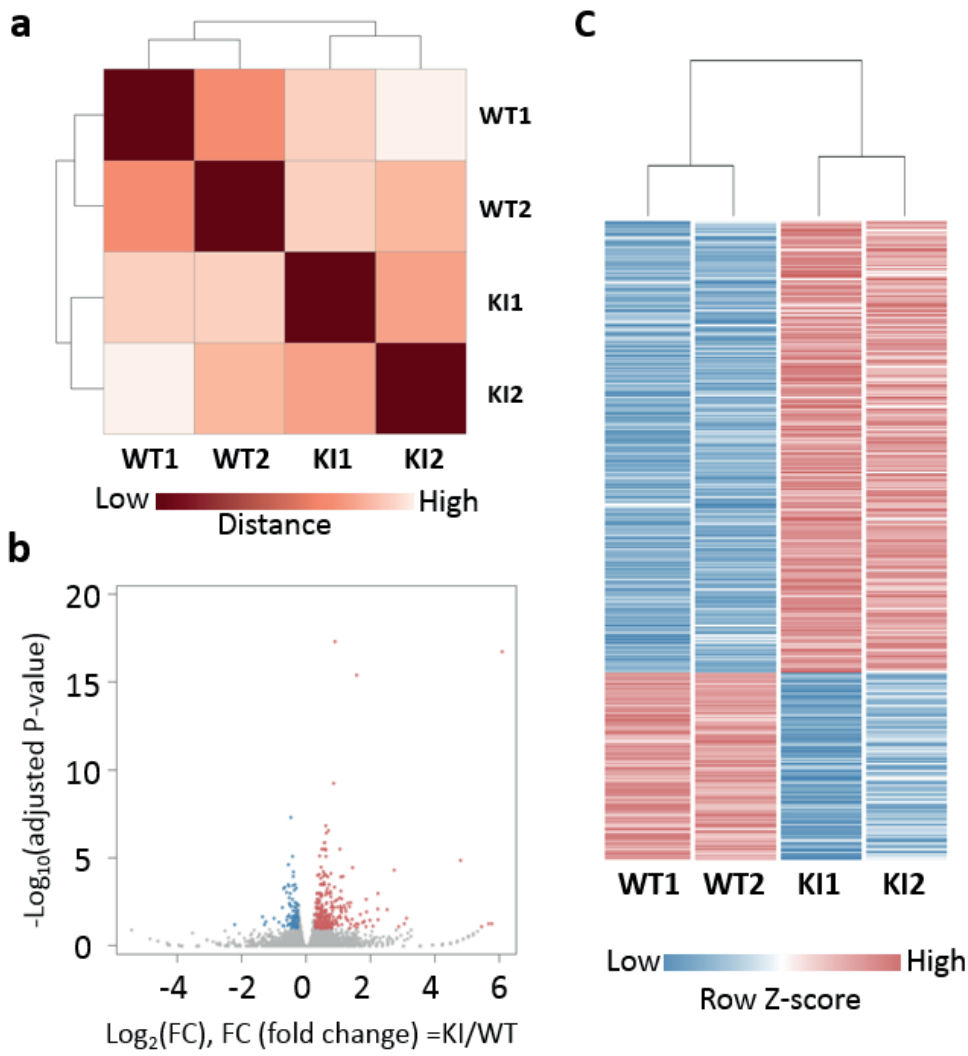
a
WT1
WT2
KI1
KI2
WT1 WT2 KI1 KI2
Low
High
Distance
b
20
15
10
5
0
-Log10(adjusted P-value)
-4 -2 0 2 4 6
Log2(FC), FC (fold change) =KI/WT
c
WT1 WT2 KI1 KI2
Low
High
Row Z-score

Figure 7. Distinctive gene expression between *Lsd1 KI* mice and WT littermates

(a) An expression heat map using the entire gene expression profiles of two genotypes (WT and KI) and their replicates (1 and 2) of the sample-to-sample distances on the matrix. **(b)** Volcano plots of differentially expressed genes (DEGs): red (upregulated) and blue (downregulated) dots. The x-axis displays the log2-transformed fold change (FC) in gene expression: FC = expression in *Lsd1* KI mice divided by that in WT littermates. The y-axis is the Benjamini-Hochberg correction's adjusted p-value (negative $\log_{10}$ transformed). **(c)** A heat map of the level of expression (Row Z score applied to log2RPKM; reads per kb of exon per million mapped reads) of DEGs in two groups and their replicates.

Table 4. RNA-seq results of postsynaptic function-related genes in *Lsd1* KI mice

Gene	Ensembl ID	Official gene symbol	Fold change (KI/WT)	adjusted P-value
CaMKII	ENSMUSG00000024617	Camk2a	0.946831602	0.868932604
	ENSMUSG00000057897	Camk2b	0.984424572	1
	ENSMUSG00000053819	Camk2d	1.493276273	0.010488565
	ENSMUSG00000021820	Camk2g	0.967544436	1
PSD-95	ENSMUSG00000020886	Dlg4	0.949109285	0.88100209
Shank	ENSMUSG00000038738	Shank1	1.014750097	1
	ENSMUSG00000037541	Shank2	0.991837936	1
	ENSMUSG00000022623	Shank3	0.979599259	1
SynGAP	ENSMUSG00000067629	Syngap1	0.910222821	0.429287085
AMPA-R	ENSMUSG00000020524	Gria1	0.94722619	0.871391628
Homer	ENSMUSG00000007617	Homer1	1.02524285	1
	ENSMUSG00000025813	Homer2	1.06976089	0.96361693
	ENSMUSG00000003573	Homer3	1.20885583	0.257945772
NMDA-R	ENSMUSG00000026959	Grin1	0.941290979	0.835275408
	ENSMUSG00000030209	Grin2b	0.895378157	0.480362467
	ENSMUSG00000002771	Grin2d	1.394298606	0.001277855
	ENSMUSG00000059003	Grin2a	0.988728908	1
	ENSMUSG00000020734	Grin2c	1.022019205	1
	ENSMUSG00000039579	Grin3a	1.034495522	1
	ENSMUSG00000035745	Grin3b	0.928031368	1
	ENSMUSG00000030209	Grin2b	0.895378157	0.480362467
Neuroligin	ENSMUSG00000063887	Nlgn1	0.878217366	0.384419499
	ENSMUSG00000051790	Nlgn2	1.046854993	1
	ENSMUSG00000031302	Nlgn3	0.909133198	0.572345631
mGluR	ENSMUSG00000019828	Grm1	1.039746035	1
	ENSMUSG00000023192	Grm2	0.846294423	0.735507555
	ENSMUSG00000003974	Grm3	0.967941469	1
	ENSMUSG00000063239	Grm4	1.353950404	0.330244133
	ENSMUSG00000049583	Grm5	0.962924536	1
GKAP	ENSMUSG00000003279	Dlgap1	0.961107197	1
	ENSMUSG00000047495	Dlgap2	0.86914685	0.191910678
	ENSMUSG00000042388	Dlgap3	0.992816067	1
	ENSMUSG00000061689	Dlgap4	1.026949569	1
SPAR	ENSMUSG00000042700	Sipa1l1	1.035550725	1
	ENSMUSG00000001995	Sipa1l2	0.818213947	0.010773749
	ENSMUSG00000030583	Sipa1l3	0.839161555	0.025267035
nNOS	ENSMUSG00000029361	Nos1	0.844197576	0.094498538
	ENSMUSG00000020826	Nos2	1.20509329	1
	ENSMUSG00000028978	Nos3	1.186078812	0.692800787
GRIP	ENSMUSG00000034813	Grip1	0.840701097	0.301190363

Table 5. Upregulated presynaptic function-related genes in *Lsd1* KI mice

Gene	Ensembl ID	Gene symbol	Fold change (KI/WT)	adjusted P-value
H1R	ENSMUSG00000053004	Hrh1	1.598754618	0.083037523
H3R	ENSMUSG00000039059	Hrh3	1.760994935	0.006266628
D2R	ENSMUSG00000032259	Drd2	1.922056591	0.044347265
VMAT2	ENSMUSG00000025094	Slc18a2	1.457457864	0.07082698

Phosphorylation of Lsd1 is required in the expression of presynaptic-function related gene

I obtained some information on differentially expressed genes in *Lsd1* KI mice from the previous RNA-seq data. To archive more specific evidence for the expression level of genes related to presynaptic plasticity and memory, I performed qRT-PCR of total hippocampal RNA. The results showed that the up-regulation of the genes *Crhr1*, *Hrh1*, *Hrh3*, *Oxtr*, *Drd2*, *Slc18a2 (VMAT2)*, *Rab39*, and *Syngr1* was congruent with the RNA-Seq analysis results (Fig. 8a). However, contrary to my expectations, there was no distinctive expression of *Bsn*, *Ppfia2 (Liprin-a-2)*, and *Rims1* between the genotypes (Fig. 8a).

In order to confirm whether the altered expression of these genes is due to PKCα-mediated phosphorylation deficiency, I designed an experiment where PKCα activity is first blocked, and then the expression level of the presynaptic function-related gene was examined. The previous study suggested that the treatment of Go6976, a PKCα inhibitor, attenuates Lsd1 phosphorylation induced by a PKCα activator (Nam, Boo et al. 2014). In this experiment, Go6976 (100 nM) was treated to a culture of primary hippocampal neurons for 8 hours, and then the mRNA levels of presynaptic function-related genes were measured. The data indicated that the treatment of Go6976 induced an increase in expression of *Crhr1*, *Hrh1*, *Hrh3*, *Oxtr*, *Drd2*, *Slc18a2 (VMAT2)*, *Rab39*, and *Syngr1* genes compared to the vehicle group. These results are in line with the results of the RNA-seq and qRT-PCR described above (Fig. 8b).

Taken together, the results of the series of experiments investigating the gene

expression profiles in *Lsd1* KI mice and WT littermates indicate that several genes involved in memory and presynaptic plasticity were regulated by PKCα-mediated phosphorylation of Lsd1.

Figure 8

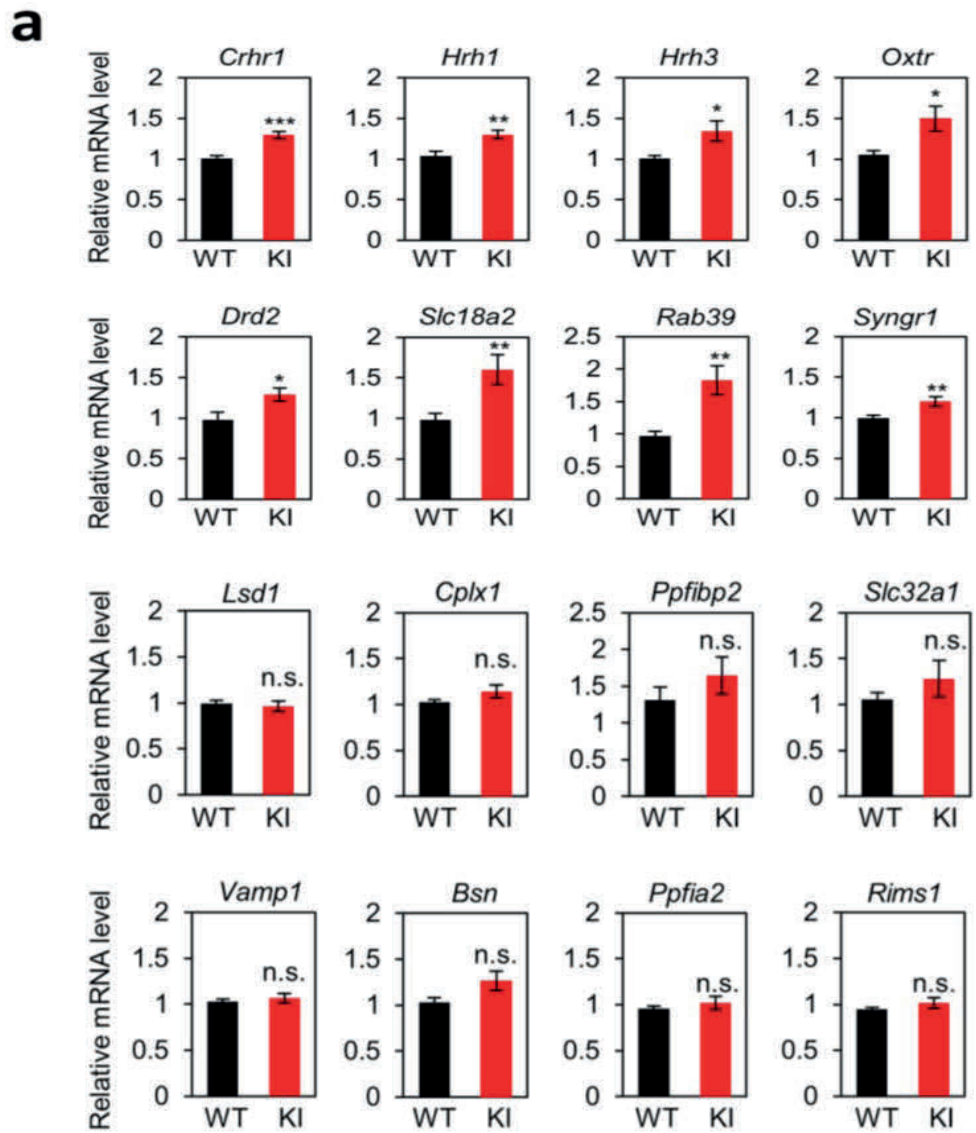
a
Crhr1
Hrh1
Hrh3
Oxtr
Drd2
Slc18a2
Rab39
Syngr1
Lsd1
Cplx1
Ppfibp2
Slc32a1
Vamp1
Bsn
Ppfia2
Rims1
Relative mRNA level
WT
KI
n.s.

Figure 8

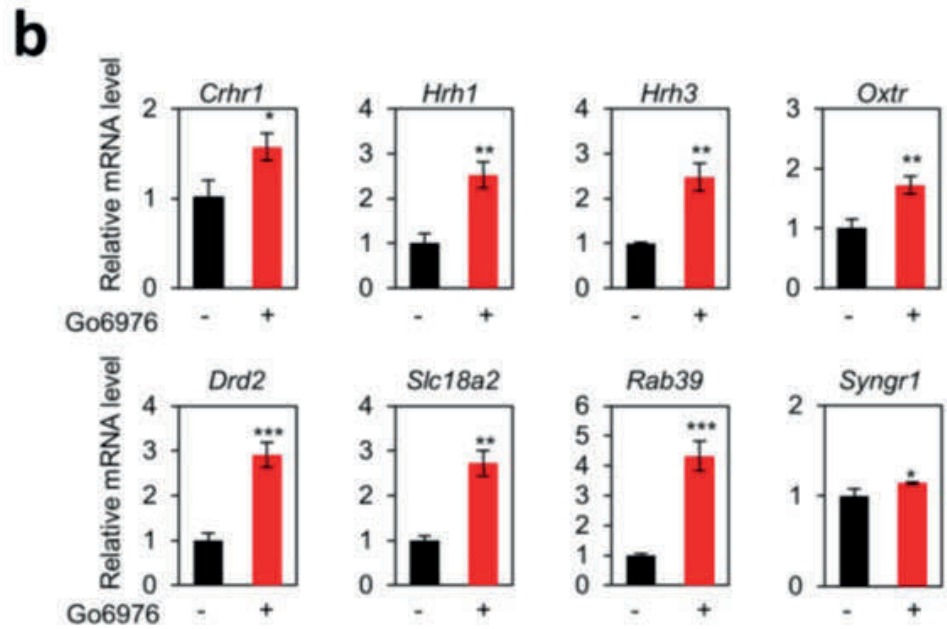

Figure 8. Phosphorylation of Lsd1 required presynaptic-function related gene expression

(a) Quantitative RT-PCR analysis (qRT-PCR). Distinctive expression of genes between the genotypes. (WT: n = 9, KI: n = 9; unpaired *t*-test, **p* < 0.05, ***p* < 0.01, ****p* < 0.001, ns: not significant). Data are expressed as mean ± SEM. (**b**) qRT-PCR analysis of hipppcampal culture after the application of a PKCα inhibitor (GM 6976 (100 nM, 8h)) (n = 3; unpaired *t*-test, **p* < 0.05, ***p* < 0.01, ****p* < 0.001). Data are expressed as mean ± SD.

DISCUSSION

In summary, phosphorylation-defective Lsd1 induced impairments in hippocampus-dependent memories such as contextual fear memory (Fig. 1b and 1c), spatial memory (Fig. 1d and Fig. 2), and social recognition memory task (Fig. 4d), but not in amygdala dependent memory (Fig. 1e).

The results of electrophysiology experiments were consistent with these findings. Although E- LTP, L-LTP, and LTD (Fig. 6) were normal in *Lsd1* KI mice, abnormal presynaptic functions such as lower PTP and PPR and increased mEPSC frequency were observed (Fig. 5). While long-term synaptic plasticity has been mainly reported as the physiological mechanism for learning and memory, several reports suggest that the regulation of associated learning and memory requires short-term synaptic plasticity. For example, Silva et al. (Silva, Rosahl et al. 1996) established that impaired learning and memory had been developed in several transgenic mouse lines such as CaMKIIα heterozygote knock-out (CaMKII-α+ /−) and Synapsin II knock-out mice. These mice displayed intact CA1 LTP but a short-term plasticity deficit in various forms. Moreover, RIM1α is a presynaptic protein that plays a role in maintaining the normal release of neurotransmitters (Schoch, Castillo et al. 2002) and long-term presynaptic potentiation (Castillo, Schoch et al. 2002). It was reported that RIM1α KO mice also showed comparable phenotypes to my results, displaying normal LTP but abnormal short-term plasticity: lower PTP and higher PPR and impairment of long-term fear memory (Powell, Schoch et al. 2004). Experiments on (SAD)-B KO mice recently demonstrated a long-term fear memory impairment, with improved PPR and lower frequency of mEPSC, but no alternation of PTP and LTP

(Watabe, Nagase et al. 2016). These pieces of evidence are consistent with the results of the current study that changes in short-term synaptic plasticity, especially presynaptic alterations, caused memory impairment. There is no convincing explanation for how alterations in these various types of presynaptic plasticity could lead, in a concerted manner, to a change in the regulation of cognition. Nevertheless, when the impacts of a number of presynaptic abnormalities are accumulated, it may be sufficient to result in an impairment of associative memory formation.

In line with this, the findings of the Genome-wide RNA-seq study on hippocampal tissues indicate that *Lsd1* KI mice exhibit altered expression levels of several genes that function in memory and presynaptic plasticity. Moreover, I confirmed that PKCα inhibitor (Go6976) treatment induced the up-regulation of presynaptic-function related genes, which were found to be up-regulated in the RNA-seq result on RNAs extracted from hippocampal tissues of *Lsd1* KI mice. These results suggest that a large assortment of genes that take a role in memory and presynaptic plasticity requires Lsd1 phosphorylation. One possible explanation for up-regulation of presynaptic function-related genes caused by phosphorylation deficient Lsd1 is that deficit in phosphorylation might alter the structure of Lsd1, which then disrupts interactions with other molecules such as HDAC, Co-rest, and Androgen receptor. Since serine 112 residue is not placed within the region of Lsd1 protein with amine oxidase activity important for the enzymatic reaction, intrinsic histone demethylase activity may not have been influenced by the change in Lsd1 phosphorylation site (Nam, Boo et al. 2014). Thus, we assume that molecular changes observed in this study might have been induced by promotion of interactions between

phosphorylated form of Lsd1 and the molecules involved in the regulation of presynaptic function-related genes. Further work will be required.

A previous study showed that phosphorylation of Lsd1 plays an important role in circadian rhythm regulation. Phosphorylation of Lsd1 induced its interaction with CLOCK:BMAL1, which then triggered the transcription mediated by the E-box. Moreover, *Lsd1* KI mice displayed disrupted circadian rhythms (Nam, Boo et al. 2014). Previously studies suggested that memory can be affected by the perturbation to the circadian clock gene. For instance, knock-out mice of the gene *Bmal1*, a critical circadian clock-related gene, showed intact anxiety-related behaviors but spatial and contextual fear memory was impaired (Wardlaw, Phan et al. 2014, Snider, Dziema et al. 2016). Thus, it is reasonable to assume that spatial memory impairment exhibited in the results of the current study might also be induced by the perturbation in circadian rhythmicity in *Lsd1* KI mice.

Taken together, in this study, I investigated the role of PKCα-mediated phosphorylation-deficit Lsd1 through behavioral experiments, electrophysiological measurements, and gene expression profiling. I found that the role of phosphorylation of Lsd1 is specific to the regulation of hippocampus-dependent learning and memory, and presynaptic plasticity.

CHAPTER III

Neurl 1 and *Neurl 2* are required for the regulation of hippocampus-dependent spatial memory and protein synthesis-dependent LTP

INTRODUCTION

Among the three types of ubiquitin enzymes, E1, E2, and E3, there exist especially various kinds of E3 ubiquitin enzymes (Zheng and Shabek 2017). Of the numerous E3 ligases, *neur*, known as *Drosophila* neurogenic gene (Lehmann, Jimenez et al. 1983, Boulianne, de la Concha et al. 1991), encodes an ubiquitin E3 ligase (Yeh, Dermer et al. 2001) composed of three domains: two copies of a novel domain, the neuralized homology repeat (NHR), and a C-terminal C3HC4 RING Zn-finger (RING) domain (Price, Chang et al. 1993, Nakamura, Yoshida et al. 1998). *Neur* is involved in Notch signaling regulation (Koutelou, Sato et al. 2008), and known to regulate long-term memory formation in *Drosophila* (Pavlopoulos, Anezaki et al. 2008, Rullinkov, Tamme et al. 2009). In rodents, *Neurl* is the mouse homolog of the *Drosophila neur* gene (Pavlopoulos, Kokkinaki et al. 2002, Song, Koo et al. 2006), and its product proteins were found to be mostly localized in neuronal dendrites, and its expression level changed upon a neuronal activity (Timmusk, Palm et al. 2002). Specifically, when *Neurl 1* was overexpressed in the mouse hippocampus, LTP and hippocampus-dependent memory were both enhanced. This memory-enhancing effect was associated with an increase in the number of synapses and AMPAR subunits, GluA1 and GluA2, by the up-regulation of a transcriptional factor, cytoplasmic polyadenylation element binding protein 3 (CPEB3) (Pavlopoulos, Trifilieff et al. 2011).

Neurl 2, a paralog of *Neurl 1* (Timmusk, Palm et al. 2002), also acts as an E3 ligase and regulates Notch signaling pathway (Rullinkov, Tamme et al. 2009). In mouse embryos, the expression pattern of *Neurl 2* was similar to that of *Neurl 1* and both

Neurl 1 and *Neurl 2* have a comparable biochemical activity such as proteasome-dependent degradation (Song, Koo et al. 2006). However, *Neurl 1* transcripts were localized in the dendrites of hippocampal neurons, while *Neurl 2* transcripts were observed in the cytoplasm of the cells (Rullinkov, Tamme et al. 2009). Also, expression of *Neurl 1* was granule cell-specific while *Neurl 2* showed Purkinje cell-specific expression (Timmusk, Palm et al. 2002).

Aforementioned studies have suggested both similarities and differences between the properties of *Neurl 1* and *Neurl 2*. However, whether the genes have overlapping functions or whether the genes have some distinctive roles, remains unknown. Here, I investigated the role of these genes in hippocampus-dependent learning and memory using *Neurl 1* knock-out (*Neurl 1* KO), *Neurl 2* knock-out (*Neurl 2* KO) and *Neurl 1* and *Neurl 2* knock-out (*Neurl 1,2* KO) mice. I revealed that spatial memory was impaired in *Neurl 1,2* KO mice but not in *Neurl 1* KO, *Neurl 2* KO, and WT littermates. In addition, I found that basal synaptic properties were unchanged, but protein synthesis-dependent long-term synaptic plasticity was impaired in *Neurl 1,2* KO mice.

EXPERIMENTAL PROCEDURES

Mice

Genetic background and generation of *Neurl 1* KO, *Neurl 2* KO, *Neurl 1,2* KO mice were previously described (Ruan, Tecott et al. 2001, Koo, Yoon et al. 2007). In brief, *Neurl 1* KO, *Neurl 2* KO, *Neurl 1,2* KO mice were generated with backgrounds of C57BL/6J. These mice are whole body knock-out mice. The deleted regions encode amino acids 218 to 574 of the murine Neurl 1 protein and amino acids 115 to 319 of the murine Neurl 2 protein. Both male and female mice with all genotypes were used in each experiment. There was no difference in their behavior, depending on sex. All tests were conducted as blind tests with respect to the information of genotypes. Animal facility controlled 12-hour light-dark cycle (lights on 9:00 a.m., lights off 9:00 p.m.) and all animals were co-housed with food and water provided *ad libitum* in temperature-controlled (approximately 24°C) conditions. This research has been permitted by the Institutional Animal Care and Use Committee of Seoul National University.

Behavioral tests

Open-field test

In this study, I used a white plexiglas box (acryl 40 × 40 × 40 cm) as an open field box. Under the dim light, Mice were placed in an empty open field box and freely explored for 30 min. Spent time in each of two different zones, central (within a 20 × 20 cm) and the peripheral zone, and the mobility of each mouse were estimated using a tracking program (EthoVision 9.0, Noldus).

Elevated zero maze test

The elevated zero maze (EZM) is a round-shaped track (60 cm diameter, 5 cm width), which is lifted 65 cm above the ground level. EZM is composed of distinct zones with or without the walls. The zones without walls are referred as open arms, and the zones with 20 cm walls are referred as closed arms. Under the bright light, mice were put in one of the closed arms of the track and freely explored the apparatus for 5 min. Their movement and spent time in each arm were estimated using a tracking program (EthoVision 9.0, Noldus).

Light-dark box test

Mice were put in a rectangular plexiglas box composed of a dark zone covered in black and a light zone illuminated by the intense light of 400 lux intensity. The zones were connected by a narrow passage sized for a single mouse. The strong light was blocked by a black plexiglas board over the dark zone, and mice were allowed to freely explore either zones through the passage between them. Trials were initiated by putting the mice into the dark zone and covering it with the blackboard. For each mouse, the time spent in the light zone (31cm × 25cm) within a 10 min period was tracked with a tracking program (EthoVision 9.0, Noldus).

Morris water maze test

A round shaped tank (140 cm diameter, 100 cm height) filled with white opaque water (21~23°C) was placed within a room with several spatial cues. During the Morris water maze task, the tank was split into four virtual quadrants and a 10 cm

diameter platform was positioned at the center of the target quadrant (TQ). Mice were handled by the experimenter for 3 min per day for five consecutive days prior to training. During the training days, mice were placed at the edge of the maze facing the inner wall of the tank and trained to reach the platform within 60 seconds. If the mice were unable to arrive at the platform in 60 seconds, they were guided to and let stay on the platform for 10 seconds on training days 1 and 2. Mice were trained four times each day, with 1 min intertrial intervals. The probe test was performed under the same conditions but without a platform on the next day after training day 5.

Contextual fear conditioning test

Mice were handled for 3 min per day for three days before the experiment. After that, in a given 180-s conditioning period, mice were permitted to explore freely in the chamber (Coulbourn Instruments), and then a foot shock (2s duration, 0.4mA intensity) was given through the floor grid. At the end of the conditioning, mice were returned to their home cages. Twenty-four hours later, mice were re-exposed to the same chamber where they previously have experienced a foot shock. Freezing behavior was automatically quantified by the Freeze Frame software.

Object location memory test

Mice were first handled for 5 minutes for five consecutive days. For the next two days, the subjects were habituated for 15 minutes in an open field chamber, which had a visual cue on one side and it was transparent on the other wall. A dim light was applied throughout the whole experiment. The next day, two identical objects were placed in the box, and mice were allowed to explore and learn the object's position

for 10 minutes. One object's location was changed to the opposite side on the following day, and mice were allowed to explore for 5 minutes each. The chamber and the objects were cleaned with distilled water (DW) and 70% ethanol (EtOH) between each trial. The experimenter manually counted the time spent by each mouse interacting with the objects.

Y-maze test

Each mouse was placed at the center of the apparatus (Plexiglass and acrylic, Y maze consist of three identical arms = 30cm × 5.5cm × 15cm) and allowed to explore the apparatus for 8 min under dim light. The mice located in the Y shape maze freely moved from one arm to another. All tasks were recorded with a digital camera placed above the apparatus, and spontaneous arm alterations were manually counted. The mice that changed the arm less than five times were excluded from the analysis.

Three-chamber test

Mice were put into a rectangular plexiglas box divided into three chambers. The chambers were connected by a passage sized for a single mouse. Mice with identical sex and age as the test mice were kept in a separate rack during the experiment and were used as stranger mice. Stranger mice were put into a cylindrical metal grid mounted with a heavy paper cup. They were then put into either the left or right corner of the chamber for 10 minutes under dim light. Meanwhile, test mice were habituated to the chamber with the grids for 10 minutes under dim light. Stranger mice habituation was done for three consecutive days prior to the test, while test mice habituation was done for two days. Trials were initiated by 10 minutes of test

mouse habituation, after which a stranger and a yellow plastic block were put into the grids for 10 minutes. The block was replaced with a new stranger for the next 10 minutes of the trial. Test mice were kept in the middle chamber by transparent walls while either a stranger or an object were introduced into the grids, and allowed to freely explore after the walls were lifted. Every dish, grid, and chamber were cleaned with 70% EtOH and DW between each trial. The experimenter manually counted the total interaction time of the test mice with each grid.

Electrophysiology

Mice were anesthetized with isoflurane and killed by decapitation in accordance with the policy and regulation approved by the Institutional Animal Care and Use Committee at Seoul National University. Transverse hippocampal slices (350 μm) were prepared using a Vibratome (Leica, VT1200S) in ice-chilled slicing solution that contained (in mM): 210 sucrose, 3 KCl, 26 NaHCO3, 1.25 NaH2PO4, 5 MgSO4, 10 D-glucose, 3 sodium ascorbate and 0.5 CaCl2, saturated with 95% O2 and 5% CO2. The slices were transferred to an incubation chamber that contained (in mM): 1.25 NaH2PO4, 2 MgSO4, 10 D-glucose, and 2 CaCl2 (carbonated with 95% O2 and 5% CO2). Slices were allowed to recover at 32-34°C for 30 minutes and then maintained at 26-28 °C for a minimum of 1 h before recordings were made.

The extracellular recording was performed in an interface chamber (Campden Instruments) maintained at 32°C and perfused continuously at 2–3 ml/min with ACSF. Standard extracellular recordings were performed in the CA1 region of hippocampal slices, as described in Park et al., 2016, to measure the slope of evoked field EPSPs (fEPSPs). Recordings were monitored and analyzed using WinLTP

(Anderson and Collingridge 2007). Two independent SCCPs were stimulated alternatively, each at a frequency of 0.1 Hz. After a stable baseline of at least 20 min, LTP was induced using TBS delivered at basal stimulus intensity. An episode of TBS comprised five bursts at 5 Hz, with each burst composed of five pulses at 100 Hz. Either an episode of TBS or a train of three TBS episodes with an interval of 10 minutes was given for LTP induction. Representative sample traces are an average of five consecutive responses, collected from typical experiments (stimulus artifacts were blanked for clarity).

Western blot analysis

The hippocampus from *Neurl 1* KO, *Neurl 2* KO, *Neurl 1,2* KO mice and WT littermate were homogenized with lysis buffer (0.5% sodium deoxycholate, 50mM pH 7.6 Tris-Cl, 1mM EDTA, 1mM DTT, 0.1% SDS, 1% NP-40, and 150Mm NaCl) containing protease inhibitor cocktail (PIC). 10 μg of each sample was loaded into 4-12% SDS-PAGE gels (Invitrogen, USA). Gel with loaded proteins was transferred to ECL membrane for 4℃ overnight. The membrane was blocked with 5% skim milk solution for 1h followed by treatment of primary antibodies: mouse anti-GAPDH (1:10000, Invitrogen), goat anti-GluA1 (1:100, Santa Cruz), mouse anti-GluA2 (1:2000, BD). Secondary antibodies were treated thereafter and were composed of goat anti-mouse (1:5000, Santa Cruz), and donkey anti-goat (1:5000, Santa Cruz).

Reverse-transcription PCR (RT-PCR)

Total RNA from the hippocampus of *Neurl 1* KO, *Neurl 2* KO, *Neurl 1,2* KO mice,

and WT littermates were extracted using TRIzol reagent (Ambion). Five hundred nanogram of extracted RNA was reverse-transcribed using random hexamer (Invitrogen) and Prime Script (TAKARA) following the manufacturers' instructions. cDNA product from each reaction served as a template for subsequent PCR amplification. PCR amplifications were conducted using specific primers for each gene.

Quantitive real-time PCR (qRT-PCR)

Gene-specific primers and TOPrealTM qPCR 2X PreMIX (SYBR Green, Enzynomics) was used for qRT-PCR. The amount of mRNA was detected using the Applied Biosystems 7300 Real-Time PCR System with SYBR Green. The qRT-PCR cycling conditions were: holding on 95 °C for 15 minutes, followed by 40 cycles of 95 °C for 10 seconds, 60 °C for 15 seconds, and 72 °C for 30 seconds. The expression level of *Neurl 1* and *Neurl 2* transcripts was normalized by *GAPDH*.

Table 6. Primer list for qRT-PCR using *Neurl 1* KO mice, *Neurl 2* KO mice, *Neurl 1,2* KO mice, and WT littermates

Neurl 1	Forward	ACCATCATGACTGAACGGGG
	Reverse	AGGCTCACAGGTGAGTTGGG
Neurl 2	Forward	GTGTCAACGATGGTGAGCCA
	Reverse	GAAGGTGCTTTCCAGAAGCTG
GAPDH	Forward	TGCACCACCAACTGCTTA
	Reverse	GGATGCAGGGATGATGTT

Statistics

For analyzing the data obtained from extracellular recordings, data were normalized to the baseline preceding TBS. Statistical significance was assessed using one-way ANOVA with post-hoc Sidak's multiple comparison test. For analyzing the data from behavioral tests (Open-field test, Elevated zero maze test, Light-dark box test, Object location memory test, Morris water maze test, and Y-maze test) and molecular experiments (Western blot analysis), one-way ANOVA test were used with post-hoc Bonferroni's multiple comparison correction to determine the statistical differences between the groups. For analyzing the data from contextual fear conditioning tests, one-way ANOVA test (with post-hoc Bonferroni's multiple comparison test) and unpaired t-test were used to determine the statistical differences between the groups. For analyzing the data from three-chamber tests, paired t-test was used to determine the statistical differences in interaction time between stranger 1 and object, or stranger 1 and stranger 2. For analyzing the data from qRT-PCR experiment, unpaired t-test was used to determine the statistical significance. The level of significance is denoted as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p< 0.0001$. GraphPad Prism 8 program was used for drawing data plots and calculating statistics. All graphs are presented as mean ± SEM.

RESULTS

Neurl 1,2 KO mice showed impaired hippocampus-dependent spatial learning and memory.

To understand the role of *Neurl 1* and *Neurl 2* in hippocampus-dependent spatial learning and memory, I performed well-established spatial memory tests: object location memory (OLM) and Morris water maze (MWM) tests.

In the OLM test, I examined whether mice recognize the fact that objects' locations are altered between trials. I first trained mice to learn the location of two objects and one object was relocated to a new position the following day (Fig. 9a). For each mouse, I quantified the exploring time of the mouse spent around the relocated object. *Neurl 1* KO and *Neurl 2* KO mice exhibited no difference in exploring time compared to WT littermates. However, *Neurl 1,2* KO mice spent less time around the relocated object (Fig. 9b).

In the MWM test, I trained mice to learn the location of the platform in the round opaque white water. During training, *Neurl 1, 2* KO mice displayed retarded learning compared to WT littermates, but not with other genotypes (Fig. 9c and 9d). In the probe test, the path tracking data for the four genotypes indicate the pattern of movement of mice of each genotype in the water maze. (Fig. 9e). In the probe test, *Neurl 1* KO mice, *Neurl 2* KO mice, and WT littermates spent more time in the target quadrant, while *Neurl 1*, *2* KO mice did not spend significantly different time in each quadrant (Fig. 9f and 9g). Moreover, *Neurl 1, 2* KO mice showed a decreased number of platform crossings (Fig. 9h) and the mean distance from platform location was significantly larger than that of WT littermates (Fig. 9i). Taken together, these

results suggest that hippocampus-dependent spatial memory was intact under the expression of either *Neurl 1* or *Neurl 2* but it was not the case when both genes were absent.

Figure 9

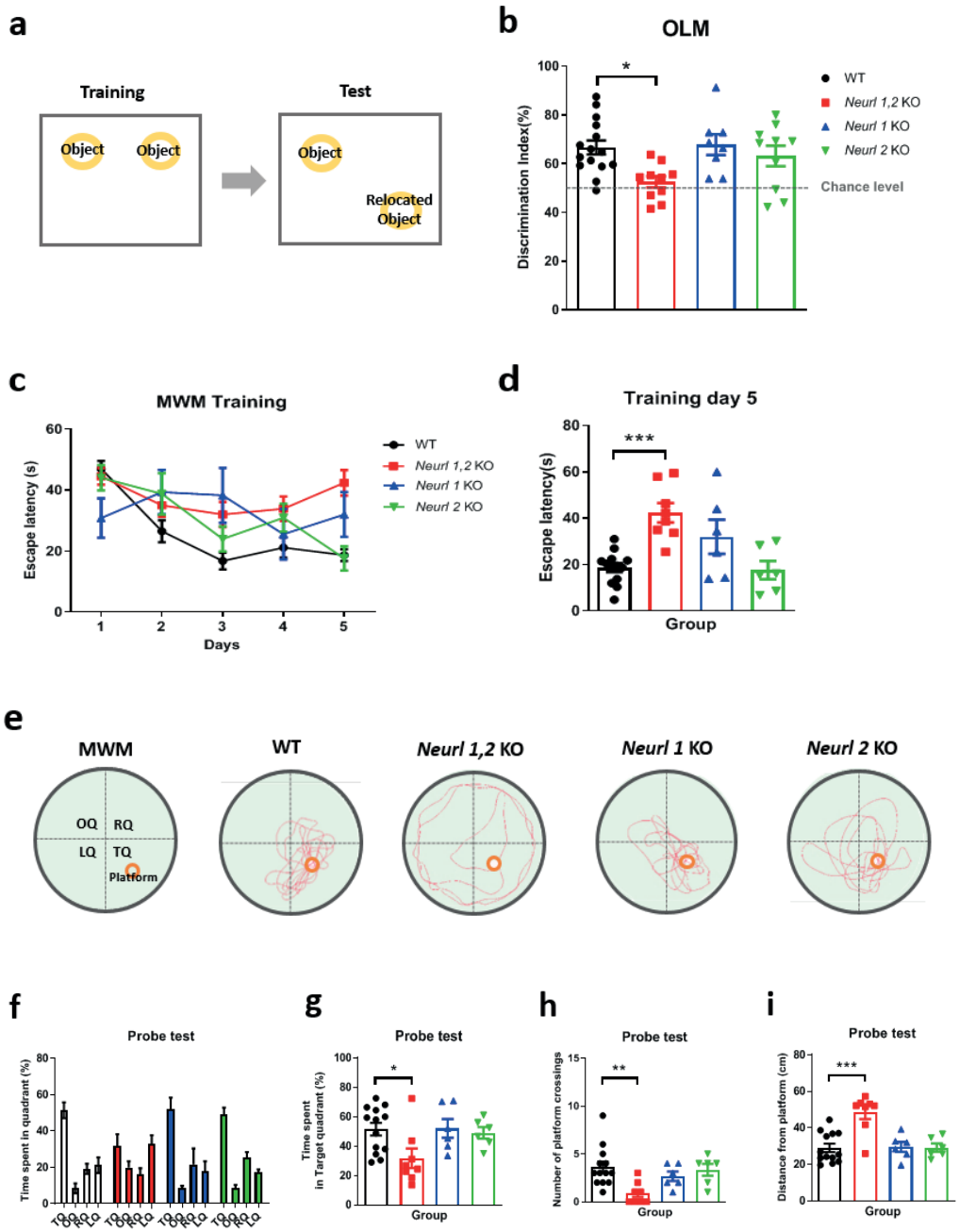
a
Training
Test
Object
Object
Object
Relocated Object
b
OLM
Discrimination Index(%)
Chance level
WT
Neurl 1,2 KO
Neurl 1 KO
Neurl 2 KO
c
MWM Training
Escape latency (s)
Days
d
Training day 5
Escape latency(s)
Group
e
MWM
WT
Neurl 1,2 KO
Neurl 1 KO
Neurl 2 KO
OQ
RQ
LQ
TQ
Platform
f
Probe test
Time spent in quadrant (%)
g
Probe test
Time spent in Target quadrant (%)
Group
h
Probe test
Number of platform crossings
Group
i
Probe test
Distance from platform (cm)
Group

Figure 9. *Neurl 1,2* KO mice showed impaired hippocampus-dependent spatial learning and memory

(a) Schematic drawings of the OLM test. **(b)** *Neurl 1,2* KO mice showed impaired object discrimination index in the OLM test. Discrimination index was calculated as follows: exploring time of relocated object / exploring time of both objects. (WT: n=15, *Neurl 1,2* KO: n=10, *Neurl 1* KO: n=8, *Neurl 2* KO: n=10; One-way ANOVA, Bonferroni's multiple comparison test, WT and *Neurl* 1,2 KO, *p<0.05). **(c)** Learning curve during 5 training days of MWM test showing the latency of the mice to reach the platform. **(d)** *Neurl 1,2* KO mice show delayed escape latency on training day 5 (One-way ANOVA, Bonferroni's multiple comparison test, WT and *Neurl* 1,2 KO ***p<0.001). **(e)** Schematic drawings of MWM test and representative path tracking data for each genotype. **(f-g)** Time spent in each quadrant during 1-minute probe test 24 h after training day 5 (One-way ANOVA of time spent in TQ, Bonferroni's multiple comparison test, WT and *Neurl* 1,2 KO, *p<0.05). **(h)** *Neurl 1,2* KO mice crossed the platform position significantly lesser compared to other groups during 1-minute probe test (One-way ANOVA, Bonferroni's multiple comparison test, WT and *Neurl* 1,2 KO **p<0.01). **(i)** *Neurl 1,2* KO mice kept a farther distance from the platform during probe test compared to WT littermates, *Neurl 1* KO, and *Neurl 2* KO mice (One-way ANOVA, Bonferroni's multiple comparison test, WT and *Neurl* 1,2 KO ***p<0.001). (WT: n=13, *Neurl 1,2* KO: n=8, *Neurl 1* KO: n=6, *Neurl 2* KO: n=6).

Neurl 1,2 KO mice showed lower, albeit not statistically significant, level of freezing in context fear conditioning

To investigate whether hippocampus-dependent associative fear memory was altered in *Neurl 1* KO, *Neurl 2* KO, and *Neurl 1,2* KO mice, I performed the contextual fear conditioning (CFC) test. I handled mice for three consecutive days, and on the following day, foot shock was given in the chamber. The next day, the mice were exposed to the identical chamber for the same duration as in the previous day fear conditioning was conducted. All genotypes showed statistically significant difference (paired t-test) between the freezing level prior to the training and during the retrieval (Fig. 10 b). In addition, I compared the freezing level of all genotypes during the retrieval; *Neurl 1,2* KO mice showed lower, albeit not statistically significant (One-way ANOVA analysis, Bonferroni's multiple comparison test), freezing level compared to WT littermates (Fig. 10 c). I also tried to analyze the effect of activity suppression, indicated by the amount of change in activity due to shock, by comparing the activity before and after receiving shock (Frankland, O'Brien et al. 2001). There was no statistically significant difference in all genotypes (Fig. 10 d).

Figure 10

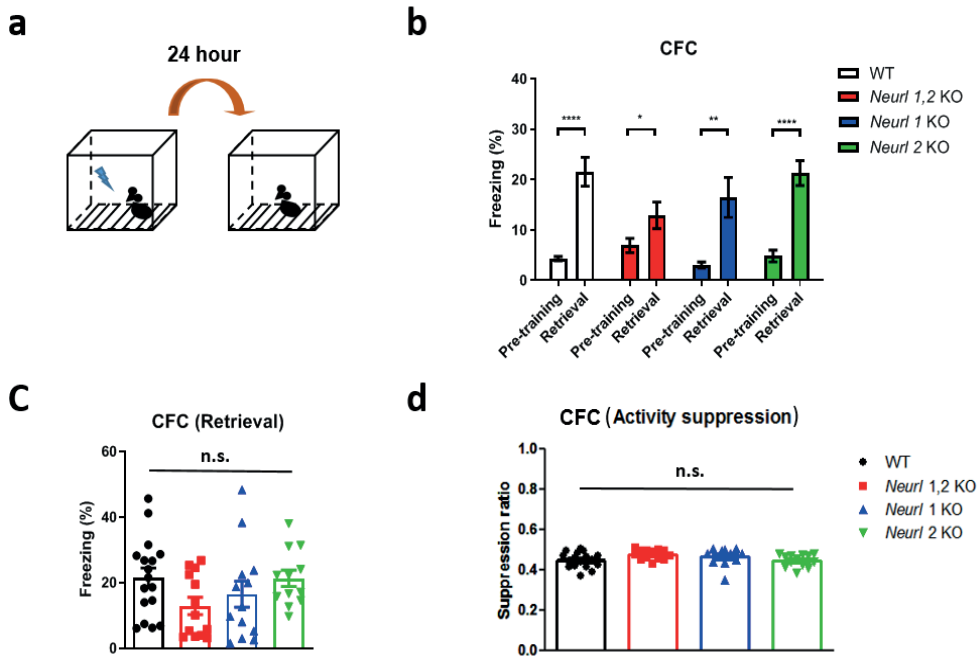
a
24 hour
b
CFC
Freezing (%)
WT
Neurl 1,2 KO
Neurl 1 KO
Neurl 2 KO
Pre-training
Retrieval
c
CFC (Retrieval)
n.s.
Freezing (%)
d
CFC (Activity suppression)
n.s.
Suppression ratio

Figure 10. *Neurl 1,2* KO mice showed lower, albeit not statistically significant, level of freezing in context fear conditioning

(a) Schematic drawings of the CFC procedure. **(b)** All genotypes displayed significantly increased levels of freezing in retrieval compared to those in pre-training (Paired t-test of pre-training and retrieval in WT group ****p <0.0001, Paired t-test of pre-training and retrieval in *Neurl 1,2* KO *p<0.05, Paired t-test of pre-training and retrieval in *Neurl 1* KO **p <0.01, Paired t-test of pre-training and retrieval in *Neurl 2* KO **** p <0.0001). **(c)** The results of the freezing level during the retrieval (One-way ANOVA, Bonferroni's multiple comparison test, p = 0.1155, n.s.: not significant, unpaired T-test of WT and *Neurl 1,2* KO, *p<0.05, unpaired T-test of WT and *Neurl 1* KO, p = 0.2959, n.s., unpaired T-test of WT and *Neurl 1* KO, p = 0.9046, n.s.). **(d)** The results of the analysis of activity suppression. The activity suppression ratio was calculated as follows: $\text{Activity}_{\text{test}} / (\text{Activity}_{\text{pre-train}} + \text{Activity}_{\text{test}})$.(One-way ANOVA, Bonferroni's multiple comparison test, p = 0.1078, n.s.). (WT: n=17, *Neurl 1,2* KO: n=13, *Neurl 1* KO: n=13, *Neurl 2* KO: n=12).

Spatial working memory was normal in all genotypes

To investigate hippocampus-dependent spatial working memory, I carried out the Y-maze task (Aggleton, Hunt et al. 1986). Mice were put into a Y-shaped maze and allowed to explore the three arms of the maze and number of spontaneous arm alternations was measured. Because of their nature for exploring novel places, mice tend to explore the most remotely visited arm rather than returning to the recently visited arms. All genotypes showed a similar level of spontaneous arm alterations (Fig. 11b). Therefore, the memory impairment of hippocampus-dependent spatial long-term memory was not due to changes in working memory.

Figure 11

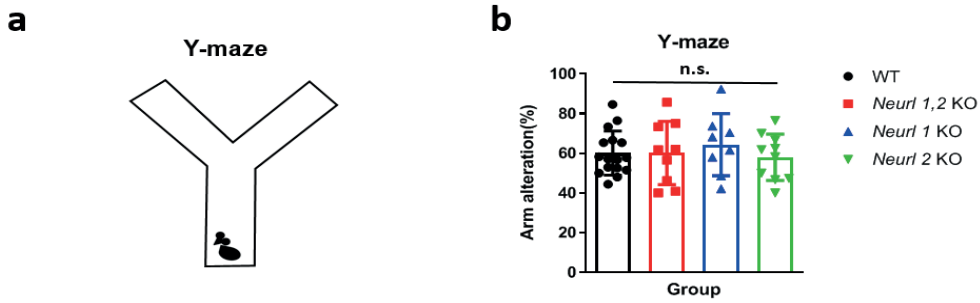

Figure 11. Spatial working memory was normal in all genotypes

(a) Schematic drawing of the Y-maze test. **(b)** All genotypes exhibited a similar level of arm alterations (WT: n=16, *Neurl 1,2* KO: n=8, *Neurl 1* KO: n=10, *Neurl 2* KO: n=9; One-way ANOVA, Bonferroni's multiple comparison test, p=0.7874, n.s.: not significant).

Social memory was normal in all genotypes

To confirm whether knock-outs of *Neurl 1* and *Neurl 2* affect the social preference and social recognition, I performed the three-chamber test. All genotypes exhibited comparable levels of interaction time in both social preference test and social recognition test (Fig. 12b and 12d). Thus, these results suggest that neither Neurl 1 nor Neurl 2 is involved in the regulation of sociability and social memory.

Figure 12

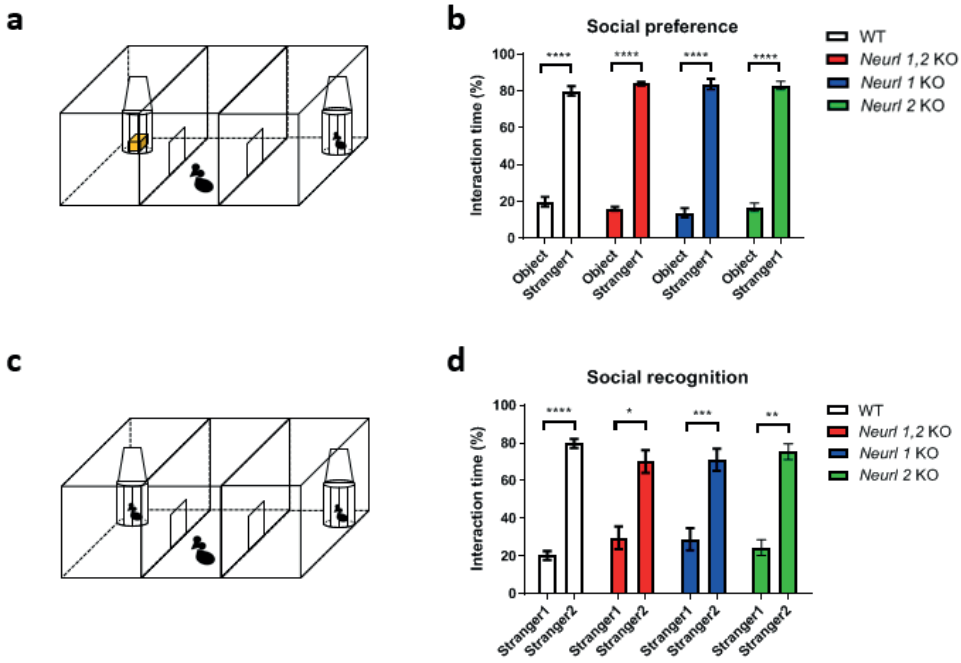
a
b
Social preference
Interaction time (%)
WT
Neurl 1,2 KO
Neurl 1 KO
Neurl 2 KO
Object
Stranger1
c
d
Social recognition
Stranger1
Stranger2

Figure 12. Social memory was normal in all genotypes

(a) Schematic drawing of social preference test. **(b)** Social preference test results (Paired *t*-test of object and stranger 1 in WT ****p<0.0001, paired *t*-test of object and stranger 1 in *Neurl 1,2* KO ****p<0.0001, paired *t*-test of object and stranger 1 in *Neurl 1* KO ****p<0.0001, paired *t*-test of object and stranger 1 *Neurl 1* KO ****p<0.0001). **(c)** Schematic drawing of social recognition test. **(d)** Social recognition test results (Paired *t*-test of stranger 1 and stranger 2 in WT ****p<0.0001, Paired *t*-test of stranger 1 and stranger 2 in *Neurl 1,2* KO *p<0.05, Paired *t*-test of stranger 1 and stranger 2 in *Neurl 1* KO ***p<0.001, Paired *t*-test of stranger 1 and stranger 2 in *Neurl 2* KO **p<0.01). (WT: n=8, *Neurl 1,2* KO: n=7, *Neurl 1* KO: n=9, *Neurl 2* KO: n=10).

Anxiety-like behavior was partially decreased in *Neurl 2* KO mice

To examine whether the knock-out of *Neurl 1* or *Neurl 2* cause changes in basal anxiety level, I employed open-field (OF) test, elevated zero maze (EZM) test, and light-dark (LD) box test, which are well-established tests for measuring anxiety-like behaviors of mice. Since their innate aversion to the highly illuminated areas, mice with a high level of anxiety prefer dark side compared to light side in the LD test. Four genotypes did not show any significant difference in EZM and LD box test (Fig. 13e and 13g), but a significantly lower level of anxiety was observed for *Neurl 2* KO mice in OF test (Fig. 13b). These results suggest that the spatial memory deficit displayed by *Neurl 1, 2* KO mice was not due to mood alteration. In addition, *Neurl 2* KO mice showed decreased anxiety-like behavior in OF test while was not impaired in hippocampus-dependent long-term memory. Thus, this decrease in anxiety level by the knock-out of *Neurl 2* did not affect hippocampus-dependent long-term memory.

Figure 13

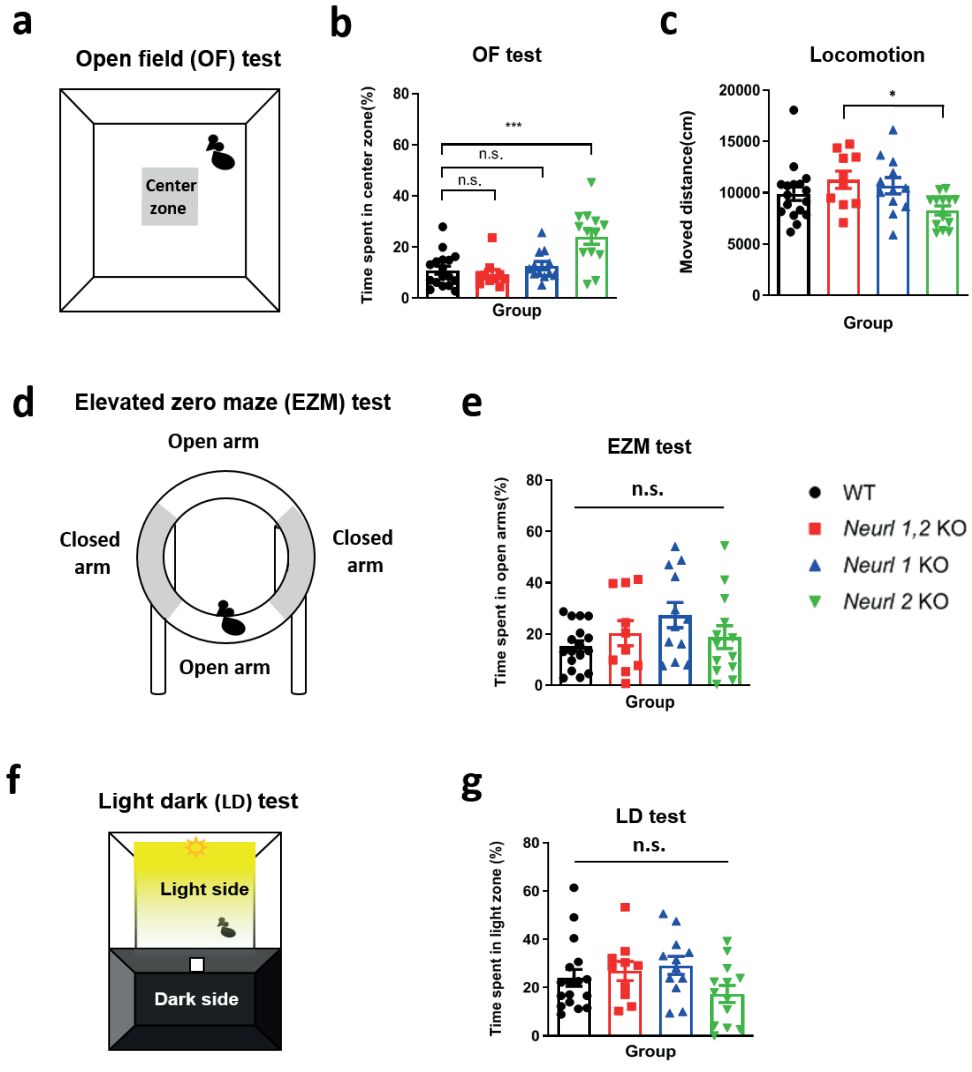
a
Open field (OF) test
Center zone
b
OF test
Time spent in center zone(%)
***
n.s.
n.s.
Group
c
Locomotion
Moved distance(cm)
*
Group
d
Elevated zero maze (EZM) test
Open arm
Closed arm
Closed arm
Open arm
e
EZM test
Time spent in open arms(%)
n.s.
Group
WT
Neurl 1,2 KO
Neurl 1 KO
Neurl 2 KO
f
Light dark (LD) test
Light side
Dark side
g
LD test
Time spent in light zone (%)
n.s.
Group

Figure 13. Anxiety-like behavior was partially decreased in *Neurl 2* KO mice

Three kinds of anxiety tests: OF test, EZM test and LD test (WT: n=17, *Neurl 1,2* KO: n=10, *Neurl 1* KO: n=12, *Neurl 2* KO: n=13). **(a)** Schematic drawing of OF test. **(b)** *Neurl 2* KO mice spent increased time in the center zone compared to other genotypes (One-way ANOVA, Bonferroni's multiple comparison test, WT and *Neurl* 2 KO ***p<0.001). **(c)** There was no significant difference in moved distances of Neurl 1,2 KO, Neurl 2 KO and Neurl 1 KO mice compared to WT littermates; however, Neurl 2 KO mice showed decreased moved distance compared to Neurl 1, 2 KO mice (One-way ANOVA, Bonferroni's multiple comparison test, Neurl 1,2 KO and Neurl 2 KO *p<0.05). **(d)** Schematic drawing of EZM test. **(e)** No group showed a significant difference in anxiety-like behavior (One-way ANOVA, Bonferroni's multiple comparison test, p = 0.1687, n.s.: not significant). **(f)** Schematic drawing of LD test. **(g)** No group displayed significant difference in anxiety-like behavior (One-way ANOVA, Bonferroni's multiple comparison test, p = 0.1523, n.s.).

Table 7. Summary of behavioral experiments with *Neurl 1* KO mice, *Neurl 2* KO mice, and *Neurl 1,2* KO mice

	Neurl 1 KO	*Neurl 2* KO	*Neurl 1,2* KO
Hippocampus-dependent spatial memory	N.S.	N.S.	Impaired
Hippocampus-dependent fear memory	N.S.	N.S.	The freezing level was lower, albeit not statistically significant
Spatial working memory	N.S.	N.S.	N.S.
Social preference & Social recognition	N.S.	N.S.	N.S.
Anxiety	N.S.	Partially decreased	N.S.

N.S.: Not Significant

L-LTP was impaired in *Neurl 1,2* KO mice

Previous studies have suggested that E3 ubiquitin ligases regulate hippocampal synaptic plasticity (Pavlopoulos, Trifilieff et al. 2011, Takagi, Setou et al. 2012, Schreiber, Vegh et al. 2015). In addition, localization of *Neurl 1* and *Neurl 2* transcripts were dissimilar within the hippocampus (Rullinkov, Tamme et al. 2009). Therefore, I conducted a series of recording experiments to find out whether the deletion of *Neurl 1* or *Neurl 2* altered basal synaptic properties in the hippocampus. I recorded the input-output (I-O) relationship and paired-pulse facilitation ratio (PPR) from SC-CA1 synapses in acute hippocampal slices of *Neurl 1* KO*, Neurl 2* KO*, Neurl 1,2* KO mice, and WT littermates. All genotypes showed intact basal synaptic properties (Fig. 14a and 14b).

Furthermore, I investigated the mechanism responsible for the hippocampus-dependent spatial memory impairment imputed to *Neurl 1* and *Neurl 2* deletion. I performed extracellular field EPSP recordings at the SC-CA1 synapses in acute hippocampal slices obtained from *Neurl 1* KO*, Neurl 2* KO*, Neurl 1,2* KO mice, and WT littermates. *Neurl 1,2* KO mice exhibited deficits in the late-phase LTP (L-LTP), but not in the early-phase LTP (E-LTP) (Fig. 14d and 14f). These findings indicate that the deletion of *Neurl 1* or *Neurl 2* does not affect basal synaptic transmission and the E-LTP but the presence of at least one of the genes is necessary for the L-LTP.

Figure 14

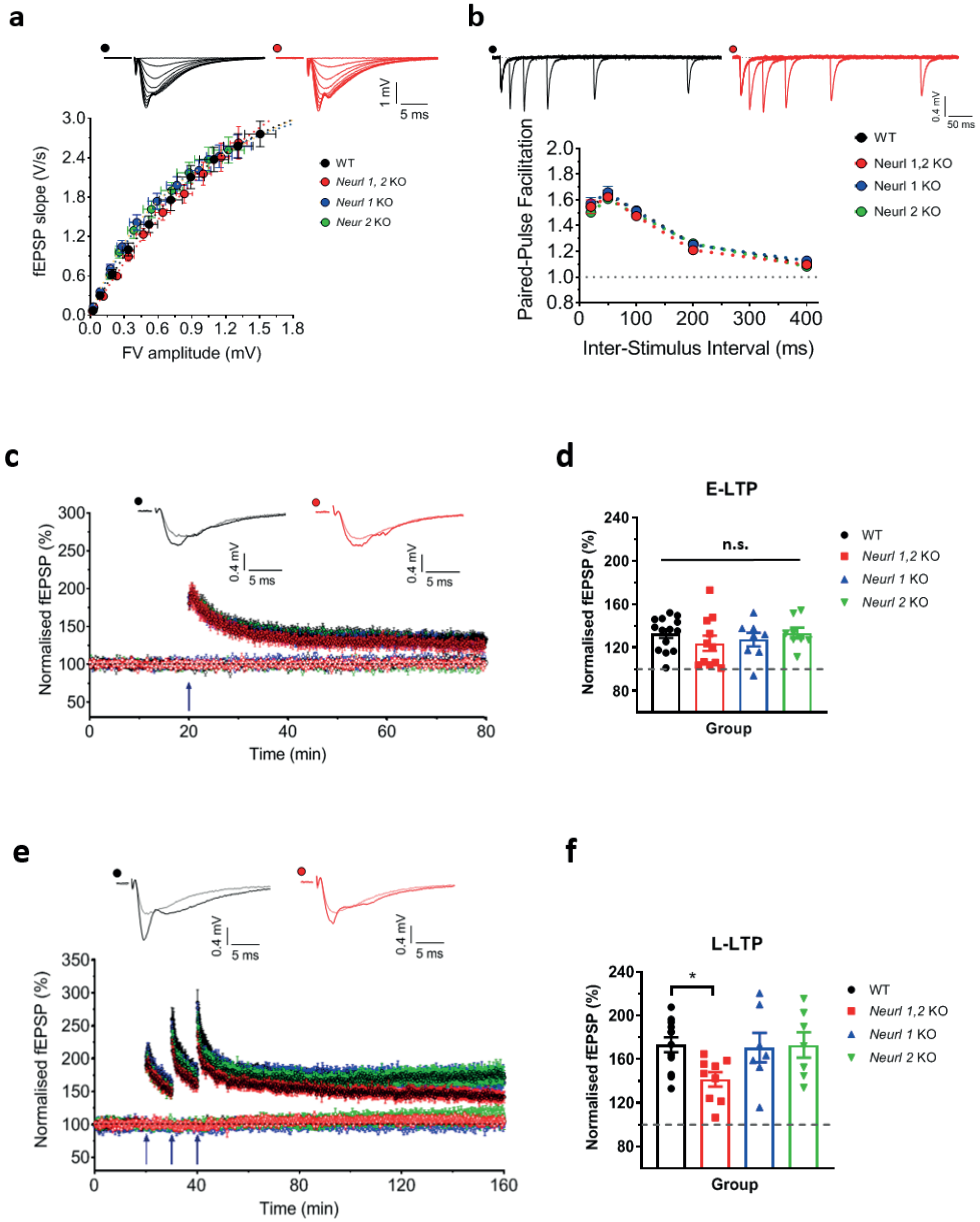
a
1 mV
5 ms
fEPSP slope (V/s)
FV amplitude (mV)
WT
Neurl 1, 2 KO
Neurl 1 KO
Neur 2 KO
b
0.4 mV
50 ms
Paired-Pulse Facilitation
Inter-Stimulus Interval (ms)
WT
Neurl 1,2 KO
Neurl 1 KO
Neurl 2 KO
c
0.4 mV
5 ms
Normalised fEPSP (%)
Time (min)
d
E-LTP
n.s.
Normalised fEPSP (%)
Group
WT
Neurl 1,2 KO
Neurl 1 KO
Neurl 2 KO
e
0.4 mV
5 ms
Normalised fEPSP (%)
Time (min)
f
L-LTP
*
Normalised fEPSP (%)
Group
WT
Neurl 1,2 KO
Neurl 1 KO
Neurl 2 KO

Figure 14. L-LTP was impaired in *Neurl 1,2* KO mice

(a-b) Results of input-output relationships and paired-pulse facilitation from SC-CA1 synapses in acute hippocampal slices from *Neurl 1* KO, *Neurl 2* KO, *Neurl 1,2* KO mice, and WT littermates. All genotypes showed no significant impairment in the basal synaptic properties (WT: n = 11, *Neurl 1,2* KO: n = 7, *Neurl 1* KO: n = 6, *Neurl 2* KO: n = 6). **(c-d)** Results of extracellular field EPSP recordings at the SC-CA1 synapses in acute hippocampal slices obtained from *Neurl 1* KO, *Neurl 2* KO, *Neurl 1,2* KO mice, and WT littermates. No group showed significant impairment in the E-LTP when it was examined using a single episode of theta-burst stimulation (WT: n = 11, *Neurl 1,2* KO: n = 7, *Neurl 1* KO: n = 6, *Neurl 2 KO*: n = 6; One-way ANOVA, Sidak's multiple comparison test, p =0.6689, n.s.). **(e-f)** Results of extracellular field EPSP recordings at the SC-CA1 synapses in acute hippocampal slices obtained from *Neurl 1* KO, *Neurl 2* KO, *Neurl 1,2* KO mice, and WT littermates. *Neurl 1,2* KO mice specifically exhibited deficits in the L-LTP induced by three episodes of theta-burst stimulation with 10 min inter-episode interval (WT: n = 11, *Neurl 1,2* KO: n = 7, *Neurl 1* KO: n = 6, *Neurl 2* KO: n = 6 ; One-way ANOVA, Sidak's multiple comparison test, WT and *Neurl* 1,2 KO *p < 0.05).

Table 8. Summary of electrophysiological experiments with *Neurl 1* KO mice, *Neurl 2* KO mice, and *Neurl 1,2* KO mice

	Neurl 1 KO	*Neurl 2* KO	*Neurl 1,2* KO
I/O relationship and PPF ratio	N.S.	N.S.	N.S.
E-LTP	N.S.	N.S.	N.S.
L-LTP	N.S.	N.S.	Impaired

N.S.: Not Significant

The expression levels of GluA 1 and GluA2 were not changed in all genotypes

I delved further into the molecular mechanism in order to figure out which molecules caused hippocampus-dependent spatial memory impairments and synaptic plasticity deficits in *Neurl 1,2* KO mice. Since *Neurl 1* overexpression has been shown to induce changes in the expression levels of AMPA receptor subunit GluA1 and GluA2 (Pavlopoulos, Trifilieff et al. 2011), I prepared hippocampal lysates from brains of *Neurl 1* KO*, Neurl 2* KO*, Neurl 1,2* KO mice, and WT littermates and performed western blot analysis for GluA1 and GluA2 levels (Fig. 15a). Four genotypes did not show any significant difference in the expression levels of GluA1 and GluA2 (Fig. 15b and 15c). These results show that the hippocampus-dependent spatial memory impairments and synaptic plasticity deficits at least in *Neurl 1,2* KO mice were not brought about by changes in the expression levels of GluA1 and GluA2.

Figure 15

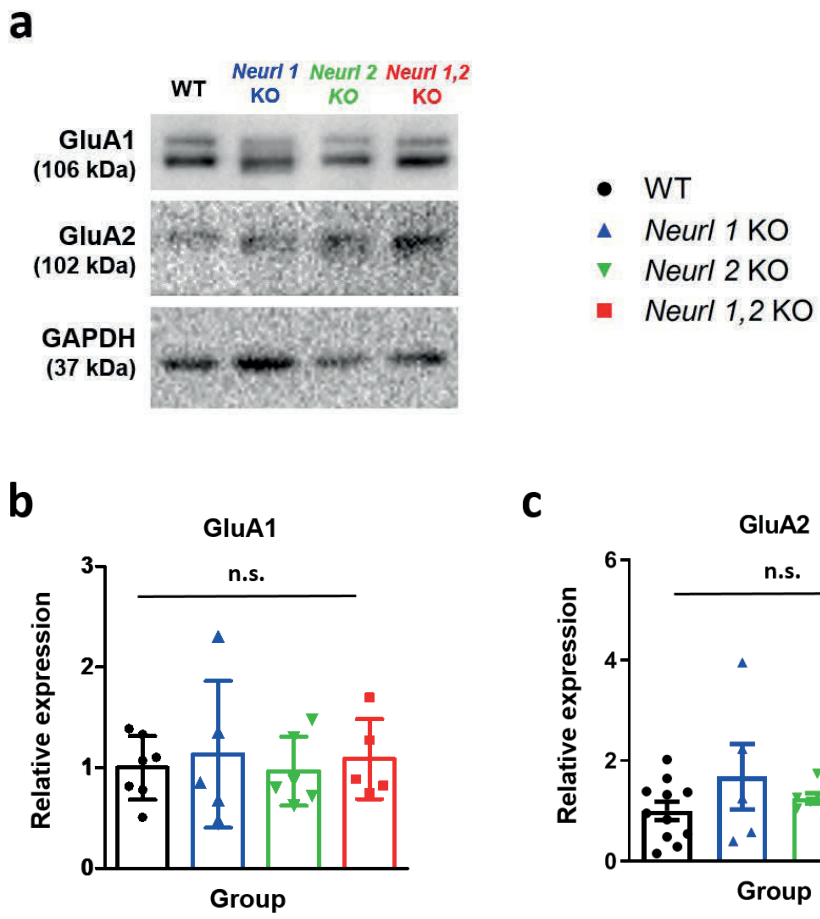
a
WT
Neurl 1 KO
Neurl 2 KO
Neurl 1,2 KO
GluA1 (106 kDa)
GluA2 (102 kDa)
GAPDH (37 kDa)
WT
Neurl 1 KO
Neurl 2 KO
Neurl 1,2 KO
b
GluA1
n.s.
Relative expression
Group
c
GluA2
n.s.
Relative expression
Group

Figure 15. The expression level of GluA1 and GluA2 was not changed in all genotypes

(a) Western blot analysis of GluA1 and GluA2 expression in the hippocampus of *Neurl 1* KO, *Neurl 2* KO, *Neurl 1,2* KO mice, and WT littermates. GAPDH was used for normalization **(b)** GluA1 levels did not significantly differ in all genotypes (WT: n = 7 mice, *Neurl 1,2* KO: n = 5 mice, *Neurl 1* KO: n = 6 mice, *Neurl 2* KO: n = 5 mice; One-way ANOVA, Bonferroni's multiple comparison test, p = 0.9227, n.s.: not significant). **(c)** GluA2 levels did not significantly differ in all genotypes. (WT: n = 11 mice, *Neurl 1,2* KO: n = 5 mice, *Neurl 1* KO: n = 6 mice, *Neurl 2* KO: n = 10 mice; One-way ANOVA, Bonferroni's multiple comparison test, p = 0.0672, n.s.).

Neurl 1 KO and *Neurl 2* KO mice exhibited no compensatory expression of *Neurl 2* and *Neurl 1* transcripts

Results reported thus far in this study suggest that hippocampus-dependent long term memory and L-LTP were impaired in *Neurl 1,2* KO but not in *Neurl 1* or *Neurl 2* single KO mice. To explain these phenomena, I hypothesized that each of *Neurl 1* and *Neurl 2* genes might play a compensatory role to each other. I expected that the gene expression level of *Neurl 1* and *Neurl 2* might have been increased in order to cover up for the absence of their respective paralog. If so, the expression of *Neurl 1* would be increased in *Neurl 2* KO mice, and vice versa. First, I performed reverse transcription-PCR (RT-PCR) with RNA molecules extracted from the hippocampus of *Neurl 1* KO, *Neurl 2* KO, *Neurl 1,2* KO mice, and WT littermates. Using specific primers for each gene, I observed *Neurl 1* transcripts were nonexistent in *Neurl 1* KO and *Neurl 1,2* KO mice, while *Neurl 2* transcripts were absent in *Neurl 2* KO and *Neurl 1,2* KO mice (Fig. 16a).

Then I conducted quantitative real-time PCR (qRT-PCR) to find out whether the expression levels of *Neurl 1* transcripts and *Neurl 2* transcripts were relatively increased in *Neurl 2* KO and *Neurl 1* KO mice, respectively, compared to WT littermates (Fig. 16b and 16c). Results showed that there was neither overexpression of *Neurl 1* transcripts in *Neurl 2* KO mice nor overexpression of *Neurl 2* transcripts in *Neurl 1* KO mice. Therefore, these results suggest that there doesn't exist a compensatory mechanism between *Neurl 1* and *Neurl 2* and moreover, either *Neurl 1* or *Neurl 2* is solely sufficient for the regulation of long-term spatial memory and L-LTP.

Figure 16

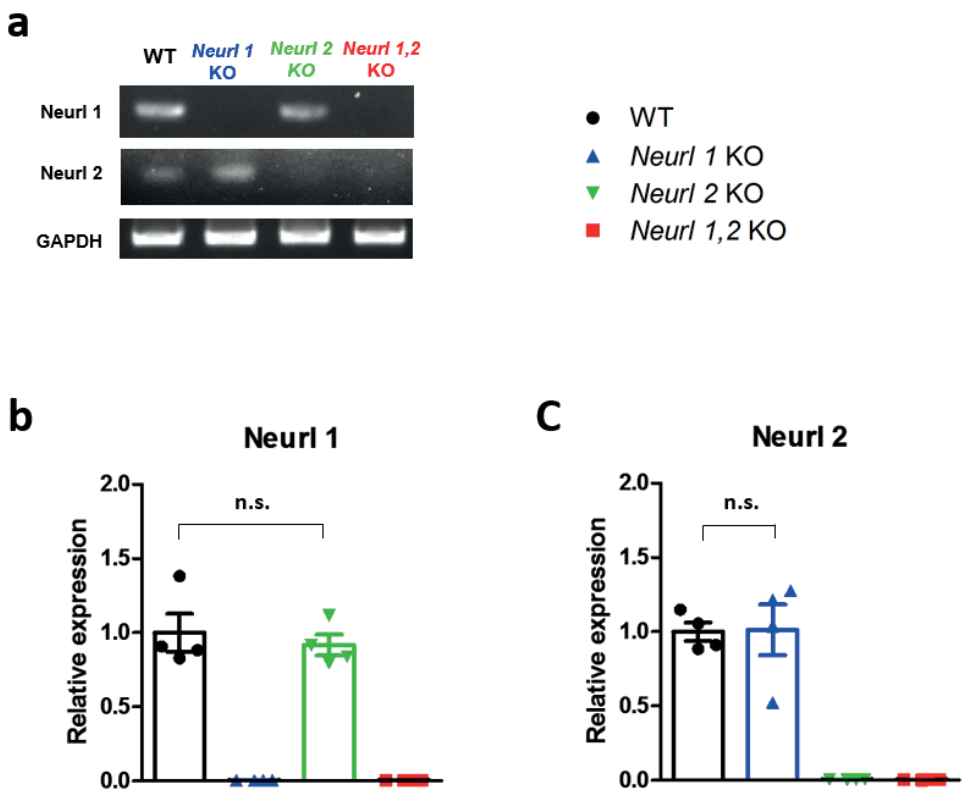
a
WT
Neurl 1 KO
Neurl 2 KO
Neurl 1,2 KO
Neurl 1
Neurl 2
GAPDH
WT
Neurl 1 KO
Neurl 2 KO
Neurl 1,2 KO
b
Neurl 1
n.s.
Relative expression
2.0
1.5
1.0
0.5
0.0
C
Neurl 2
n.s.
Relative expression
2.0
1.5
1.0
0.5
0.0

Figure 16. *Neurl 1* KO and *Neurl 2* KO mice exhibited no compensatory expression of *Neurl 2* and *Neurl 1* transcripts

(a) RT-PCR analysis showed either presence or loss of *Neurl 1* and *Neurl 2* transcripts in *Neurl 1* KO*, Neurl 2* KO*, Neurl 1,2* KO mice, and WT littermates. (WT: n=4, *Neurl 1,2* KO: n=4, *Neurl 1* KO: n=4, *Neurl 2* KO: n=4). **(b-c)** Expression levels of *Neurl 1* transcripts and *Neurl 2* transcripts were normalized by *GAPDH*. **(b)** A similar level of *Neurl 1* transcripts were observed in WT and *Neurl 2* KO mice (Unpaired T-test of WT and *Neurl 2* KO, p = 0.5924, n.s.: not significant). **(c)** A comparable level of *Neurl 2* transcripts were observed in WT and *Neurl 1* KO mice (Unpaired T-test of WT and *Neurl 1* KO, p = 0.9442, n.s.).

DISCUSSION

Previous reports have suggested that it is possible for paralogous genes to function interchangeably (Pinne, Denker et al. 2006, Khoriaty, Hesketh et al. 2018, O'Callaghan, Zarb et al. 2018), or assume disparate roles (Noree, Sirinonthanawech et al. 2019). In this study, I focused on hippocampus-dependent spatial memory and its impairment was observed in one condition in which both *Neurl 1* and *Neurl 2* were absent while it was intact when either *Neurl 1* or *Neurl 2* was single knocked-out (Fig. 9). The qRT-PCR experiment results showed that neither relative expression level of *Neurl 1* transcripts was increased in *Neurl 2* KO mice, nor that of *Neurl 2* transcripts was increased in *Neurl 1* KO mice (Fig. 15). Therefore, I suggest that there are no compensatory mechanisms between *neurl 1* and *neurl 2,* and either presence of *Neurl 1* or that of *Neurl 2* is sufficient for spatial learning and memory; furthermore I suppose neither *Neurl 1* nor *Neurl 2* is involved in spatial working memory, fear memory and social memory. Thus, it seems reasonable to assume from my findings that *Neurl 1* and *Neurl 2* perform generally comparable functionalities in the biological system. Therefore, this study provides a novel understanding regarding the functions of *Neurl 1* and *Neurl 2*, that the pair of paralogs play similar roles in hippocampus-dependent spatial memory.

I also discovered that basal synaptic transmission and E-LTP were intact in all genotypes, but L-LTP was impaired in *Neurl 1,2* KO mice. It has been widely accepted that L-LTP requires de novo protein synthesis, but such synthesis is not needed for E-LTP, which instead requires modification of existing proteins and their trafficking at synapses (Bliss and Collingridge 1993, Frey and Morris 1997, Malenka

and Bear 2004). Notwithstanding the fact that AMPA Receptor subunit GluA1 and GluA2 are important for L-LTP, the expression levels of GluA1 and GluA2 had not been altered in *Neurl 1,2* KO mice. Accordingly, I conclude that L-LTP impairment in *Neurl 1,2* KO mice was not caused by the alteration in levels of GluA1 and GluA2. Instead, I propound three alternative explanations by which deletion of *Neurl 1* and *Neurl 2* could have induced hippocampus-dependent memory and L-LTP impairment. Each of the alternative explanations considers the role of one of three different types of substrates of Neurl 1 and Neurl 2: CPEB3, Notch ligands, and cGMP-specific phosphodiesterase 9A (PDE9A).

First, *Neurl 1* regulates LTP and LTD maintenance through mono-ubiquitinated CPEB3, which promotes the production of AMPA receptor subunit GluA1 and GluA2 (Pavlopoulos, Trifilieff et al. 2011). However, the expression levels of GluA1 and GluA2 were unchanged in all the genotypes (Fig.15). Therefore, I assumed that the possibility is slim to none that hippocampus-dependent memory and L-LTP impairment were brought by the deficit of mono-ubiquitinated CPEB3 in *Neurl 1,2* KO mice. Second, *Neurl 1* and *Neurl 2* are involved in the regulation of Notch signaling pathway. *Neurl 1* represses Notch signaling by down-regulating the expression of Notch ligand Jagged1 (Koutelou, Sato et al. 2008). Furthermore, *Neurl 2* regulates the endocytosis of Notch ligand Delta in cooperation with Mind Bomb-1 (Song, Koo et al. 2006). Previous reports suggested that Notch signaling pathway regulates hippocampal synaptic plasticity such as L-LTP and long-term memory formation (Wang, Chan et al. 2004, Brai, Marathe et al. 2015, Tu, Zhu et al. 2017). Therefore, it is a possibility that hippocampus-dependent memory and L-LTP

impairment might have occurred due to abnormal regulation of Notch signaling.Third, recent studies suggested that *Neurl 1* and *Neurl 2* can promote polyubiquitination of PDE9A which then leads to its proteasome-mediated degradation (Taal, Tuvikene et al. 2019). PDE9A is an enzyme one of whose functions is breaking down cGMP. Moreover, cGMP/PKG/CREB pathway is known to play a role in learning and memory. Accordingly, previous studies reported that L-LTP and long-term memory formation were enhanced by inhibition of PDE9 (van der Staay, Rutten et al. 2008) and inhibition of PDE9A rescued memory deficit (Kleiman, Chapin et al. 2012). Therefore, I lastly hypothesize that hippocampus-dependent memory and L-LTP impairment could have occurred because PDE9A was not degraded in the absence of both Neurl 1 and Neurl 2 and that this further promoted breakdown of cGMP and finally, PKG/CREB pathways were not activated (Fig. 17).

Figure 17

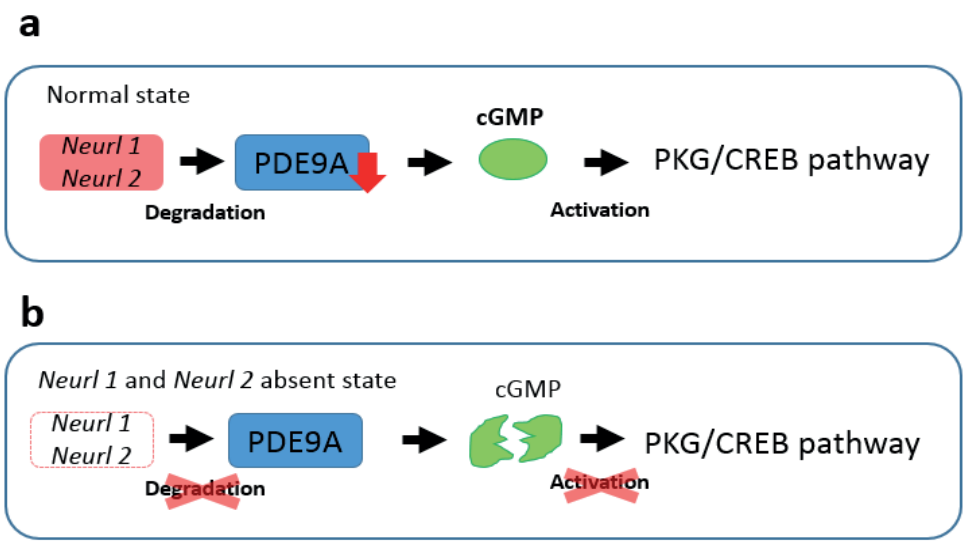

Figure 17. A model for the interaction of Neurl 1 and Neurl 2 with PDE9A in the regulation of PKG/CREB pathway

(a) In normal state, Neurl 1 and Neurl 2 promote the degradation of PDE9A through polyubiquitination of PDE9A and therefore the breakdown of cGMP is controlled under a certain level. **(b)** Under the absence of both Neurl 1 and Neurl 2, PDE9A is not degraded and cGMP molecules break down, and thereby PKG/CREB pathway cannot be activated.

Taken together, these discussions provide possible explanations for the data observed in the current study in terms of interactions between *Neurl 1* and *Neurl 2* and their substrates; that those interactions are involved in the regulation of hippocampus-dependent memory and synaptic plasticity. Further work will be required to dissect the specific functions of downstream molecules, including those mentioned above.

Pavlopoulos et al. showed impaired LTP and spatial memory in Neurl 1 inhibited mice (Pavlopoulos, Trifilieff et al. 2011). However, this result is somewhat incongruent with the results obtained in this study where *Neurl 1* KO mice had both intact LTP and spatial learning. However, another prior study reported that *Neurl 1* KO mice showed hypersensitivity to ethanol and defective olfactory discrimination while spatial memory was undamaged (Ruan, Tecott et al. 2001). I assume this difference in observed phenotypes was caused by a difference in methodologies employed in the aforementioned studies (El-Brolosy and Stainier 2017). For instance, PKMζ is a well-known molecule that plays an important role in LTP maintenance and spatial memory (Sacktor 2008). However, Tsokas et al. suggested that late-LTP and spatial memory were intact in PKMζ-null mice due to the fact that PKCι/λ compensated for the absence of PKMζ. It was also observed that the level of the compensatory protein was persistently up-regulated throughout the period of LTP maintenance (Tsokas, Hsieh et al. 2016). However, in the research cited above, Pavlopoulos et al. inhibited the expression of *Neurl 1* by expressing a dominant-negative form of the gene at a particular time point, whereas in the study of Ruan et al. and in my study, *Neurl 1* was knocked-out genetically at the embryonic stage.

Thus, I conjecture that intact spatial memory found in the current study is due to a biological compensation mechanism that functioned during the developmental stage that can substitute for the loss of *Neurl 1*.

Results obtained from anxiety behavior tests provide that *Neurl 2* KO mice, but not those of other genotypes, exhibited a significantly lower level of anxiety in OF test. However, this trend has not extended to the other anxiety tests (Fig. 12 a). Relevant to this observation, some studies provided that an experimental treatment does not always induce the same effects on these tests. It is possible that an identical treatment produce observable difference in anxiety level only in one of the tests (Paylor, Nguyen et al. 1998, Malleret, Hen et al. 1999) or even produce opposite effects across different tests (Rochford, Beaulieu et al. 1997, Strohle, Poettig et al. 1998). Although these tests are commonly based on a natural conflict within-subject animals between the drives for exploring new environments and tendencies to avoid places which are potentially dangerous, it was observed that large inter-test variations are induced under differential gene expressions and under the effect of anxiolytic drugs (Clement, Calatayud et al. 2002, Ramos 2008). Based on these observations, it could be posited that significantly lower level of anxiety in OF test was observed only in *Neurl 2* KO mice, but not in the other genotypes, since such phenotype is sufficiently caused by the existence of *Neurl 1* gene, but not so by the fact that *Neurl 2* gene does not exist, in *Neurl 2* KO mice.

Traditionally, the hippocampus has been widely held to function in spatial informational processing (O'Keefe and Nadel 1978, O'Keefe and Burgess 1996). To assess hippocampus-dependent spatial memory, I used three different memory tasks:

OLM test, MWM test, and CFC test. As a result, *Neurl 1,2* KO mice displayed dramatic impairment in spatial memory (OLM and MWM test), while exhibited lower, albeit not statistically significant, contextual fear memory. Previous studies have also observed different tendencies resulted in these two memory tests conducted against same mutant mice. For instance, mice sometimes showed intact fear memory but impaired spatial memory (Beach, Hawkins et al. 1995, Kubota, Murakoshi et al. 2001), or vice versa (Blaeser, Sanders et al. 2006, d'Isa, Clapcote et al. 2011). One possible explanation that can be provided in accounting for the results mentioned above is that the hippocampus functions in various and independent ways and these functions collectively underlie spatial memory and contextual fear memory. For example, in the MWM test, the escape location should be computed and remembered relying on the distal cues attained from the surrounding environment. In addition, a goal-directed navigation strategy is crucial in this process (Cornwell, Johnson et al. 2008, Eichenbaum 2017). However, CFC test is a type of Pavlovian fear conditioning during which a link between context and emotion is formed (Kim and Jung 2006), and this process duly requires associative learning (Brasted, Bussey et al. 2003). Therefore, I assume the test-variation observed in the results of MWM, OLM, and CFC test is due to the fact that hippocampus regulates each type of memory in a distinct manner.

CHAPTER IV

CONCLUSION

In the present study, I demonstrated two lines of evidence supporting the involvement of post-translational modification in the regulation of learning and memory, one about the effect of PKCα mediated-phosphorylation of Lsd1, and the other concerning the role of E3 ubiquitin ligases, *Neurl 1* and *Neurl 2*.

In Chapter II, I provided the first piece of evidence that supports the involvement of PKCα-mediated phosphorylation of Lsd1 in the regulation of hippocampus-dependent memory and short-term synaptic plasticity. *Lsd1* KI mice showed impairment of hippocampus-dependent fear and spatial memory. Moreover, *Lsd1* KI mice showed alteration of short-term synaptic plasticity and presynaptic function; however, long-term synaptic plasticity including LTP and LTD was normal. Providing some support to the findings just mentioned, I found that several presynaptic function-related genes are upregulated by phosphorylation-defective Lsd1.

In Chapter III, I have elucidated specific functions of *Neurl 1* and *Neurl 2* in hippocampus-dependent learning and memory. I revealed that hippocampus-dependent spatial learning and memory and protein synthesis-dependent LTP were impaired in the absence of both *Neurl 1* and *Neurl 2* but not under the presence of either *Neurl 1* or *Neurl 2*. Furthermore, I found that there existed no mechanisms between *Neurl 1* and *Neurl 2* genes for compensating one another in terms of their transcriptional level when one of the genes was absent.

Even though it is hard to say that the three genes dealt in this study, *Lsd1*, *Neurl 1*, and *Neurl 2* share the same molecular pathway in the regulation of learning and memory, these studies provide multi-faceted evidence for the fact that various forms

of post-translational modifications can work as multiple channels through which learning and memory could be controlled in its finest details.

Despite multiple experiments, the present study mainly provides observations regarding biological phenomena rather than specific mechanisms. Thus, a number of subsequent questions still remains. For instance: how phosphorylation defective *Lsd1* up-regulate presynaptic function-related genes? Which downstream molecules interact with *Neurl 1* and *Neurl 2* for regulating memories? Therefore, in further studies, it will be required to reveal more specific mechanisms and to deeply understand respective impairment of hippocampus-dependent memory observed in each transgenic mice.

Collectively, hereby presented studies add novel pieces of evidence to the understanding of the role of post-translational modifications in the regulation of learning and memory.

REFERENCE

Abel, T., P. V. Nguyen, M. Barad, T. A. Deuel, E. R. Kandel and R. Bourtchouladze (1997). "Genetic demonstration of a role for PKA in the late phase of LTP and in hippocampus-based long-term memory." Cell **88**(5): 615-626.

Aggleton, J. P., P. R. Hunt and J. N. Rawlins (1986). "The effects of hippocampal lesions upon spatial and non-spatial tests of working memory." Behav Brain Res **19**(2): 133-146.

Anderson, W. W. and G. L. Collingridge (2007). "Capabilities of the WinLTP data acquisition program extending beyond basic LTP experimental functions." J Neurosci Methods **162**(1-2): 346-356.

Ardito, F., M. Giuliani, D. Perrone, G. Troiano and L. Lo Muzio (2017). "The crucial role of protein phosphorylation in cell signaling and its use as targeted therapy (Review)." Int J Mol Med **40**(2): 271-280.

Bannister, A. J. and T. Kouzarides (2011). "Regulation of chromatin by histone modifications." Cell Res **21**(3): 381-395.

Barnes, C. A. (1979). "Memory deficits associated with senescence: a neurophysiological and behavioral study in the rat." J Comp Physiol Psychol **93**(1): 74-104.

Beach, M. E., R. D. Hawkins, M. Osman, E. R. Kandel and M. Mayford (1995). "Impairment of Spatial but Not Contextual Memory in Camkii Mutant Mice with a Selective Loss of Hippocampal Ltp in the Range of the Theta-Frequency." Cell **81**(6): 905-915.

Bear, M. F. and R. C. Malenka (1994). "Synaptic plasticity: LTP and LTD." Curr Opin Neurobiol **4**(3): 389-399.

Blaeser, F., M. J. Sanders, N. Truong, S. Ko, L. J. Wu, D. F. Wozniak, M. S. Fanselow, M. Zhuo and T. A. Chatila (2006). "Long-term memory deficits in Pavlovian fear conditioning in Ca2+/calmodulin kinase kinase alpha-deficient mice." Molecular and Cellular Biology **26**(23): 9105-9115.

Bliss, T. V. and G. L. Collingridge (1993). "A synaptic model of memory: long-term potentiation in the hippocampus." Nature **361**(6407): 31-39.

Boulianne, G. L., A. de la Concha, J. A. Campos-Ortega, L. Y. Jan and Y. N. Jan (1991). "The Drosophila neurogenic gene neuralized encodes a novel protein and is expressed in precursors of larval and adult neurons." EMBO J **10**(10): 2975-2983.

Brady, S. T. and G. J. Siegel (2012). Basic neurochemistry : principles of molecular, cellular and medical neurobiology. Amsterdam, Elsevier Academic Press.

Brai, E., S. Marathe, S. Astori, N. B. Fredj, E. Perry, C. Lamy, A. Scotti and L. Alberi (2015). "Notch1 Regulates Hippocampal Plasticity Through Interaction with the Reelin Pathway, Glutamatergic Transmission and CREB Signaling." Front Cell Neurosci **9**: 447.

Brandeis, R., Y. Brandys and S. Yehuda (1989). "The use of the Morris Water Maze in the study of memory and learning." Int J Neurosci **48**(1-2): 29-69.

Brandon, E. P., M. Zhuo, Y. Y. Huang, M. Qi, K. A. Gerhold, K. A. Burton, E. R. Kandel, G. S. McKnight and R. L. Idzerda (1995). "Hippocampal long-term depression and depotentiation are defective in mice carrying a targeted disruption of the gene encoding the RI beta subunit of cAMP-dependent protein kinase." Proc Natl Acad Sci U S A **92**(19): 8851-8855.

Brasted, P. J., T. J. Bussey, E. A. Murray and S. P. Wise (2003). "Role of the hippocampal system in associative learning beyond the spatial domain." Brain **126**(Pt 5): 1202-1223.

Castillo, P. E., S. Schoch, F. Schmitz, T. C. Sudhof and R. C. Malenka (2002). "RIM1 alpha is required for presynaptic long-term potentiation." Nature **415**(6869): 327-330.

Chakraborty, M., B. K. Paul, T. Nayak, A. Das, N. R. Jana and S. Bhutani (2015). "The E3 ligase ube3a is required for learning in Drosophila melanogaster." Biochemical and Biophysical Research Communications **462**(1): 71-77.

Clement, Y., F. Calatayud and C. Belzung (2002). "Genetic basis of anxiety-like behaviour: a critical review." Brain Res Bull **57**(1): 57-71.

Collingridge, G. L., S. Peineau, J. G. Howland and Y. T. Wang (2010). "Long-term depression in the CNS." Nat Rev Neurosci **11**(7): 459-473.

Cornwell, B. R., L. L. Johnson, T. Holroyd, F. W. Carver and C. Grillon (2008). "Human hippocampal and parahippocampal theta during goal-directed spatial

navigation predicts performance on a virtual Morris water maze." J Neurosci **28**(23): 5983-5990.

d'Isa, R., S. J. Clapcote, V. Voikar, D. P. Wolfer, K. P. Giese, R. Brambilla and S. Fasano (2011). "Mice lacking Ras-GRF1 show contextual fear conditioning but not spatial memory impairments: convergent evidence from two independently generated mouse mutant lines." Frontiers in Behavioral Neuroscience **5**.

Deribe, Y. L., T. Pawson and I. Dikic (2010). "Post-translational modifications in signal integration." Nat Struct Mol Biol **17**(6): 666-672.

Eichenbaum, H. (2017). "The role of the hippocampus in navigation is memory." J Neurophysiol **117**(4): 1785-1796.

El-Brolosy, M. A. and D. Y. R. Stainier (2017). "Genetic compensation: A phenomenon in search of mechanisms." PLoS Genet **13**(7): e1006780.

Fanselow, M. S. (2000). "Contextual fear, gestalt memories, and the hippocampus." Behav Brain Res **110**(1-2): 73-81.

Feng, J. X., G. Y. Xu, J. W. Liu, N. Zhang, L. L. Li, J. F. Ji, J. C. Zhang, L. Zhang, G. N. Wang, X. L. Wang, J. Tan, B. Q. Huang, J. Lu and Y. Zhang (2016). "Phosphorylation of LSD1 at Ser112 is crucial for its function in induction of EMT and metastasis in breast cancer." Breast Cancer Research and Treatment **159**(3): 443-456.

Fioravante, D. and W. G. Regehr (2011). "Short-term forms of presynaptic plasticity." Curr Opin Neurobiol **21**(2): 269-274.

Frankland, P. W., C. O'Brien, M. Ohno, A. Kirkwood and A. J. Silva (2001). "Alpha-CaMKII-dependent plasticity in the cortex is required for permanent memory." Nature **411**(6835): 309-313.

Frey, U. and R. G. Morris (1997). "Synaptic tagging and long-term potentiation." Nature **385**(6616): 533-536.

Giese, K. P., N. B. Fedorov, R. K. Filipkowski and A. J. Silva (1998). "Autophosphorylation at Thr286 of the alpha calcium-calmodulin kinase II in LTP and learning." Science **279**(5352): 870-873.

Gupta-Agarwal, S., T. J. Jarome, J. Fernandez and F. D. Lubin (2014). "NMDA receptor- and ERK-dependent histone methylation changes in the lateral amygdala

bidirectionally regulate fear memory formation." Learn Mem **21**(7): 351-362.

Gupta, S., S. Y. Kim, S. Artis, D. L. Molfese, A. Schumacher, J. D. Sweatt, R. E. Paylor and F. D. Lubin (2010). "Histone methylation regulates memory formation." J Neurosci **30**(10): 3589-3599.

Haas, K. F. and K. Broadie (2008). "Roles of ubiquitination at the synapse." Biochim Biophys Acta **1779**(8): 495-506.

Hasegawa, Y., D. Yoshida, Y. Nakamura and S. Sakakibara (2014). "Spatiotemporal distribution of SUMOylation components during mouse brain development." J Comp Neurol **522**(13): 3020-3036.

Hay, R. T. (2005). "SUMO: a history of modification." Mol Cell **18**(1): 1-12.

Hershko, A. and A. Ciechanover (1998). "The ubiquitin system." Annu Rev Biochem **67**: 425-479.

Hicke, L. (2001). "Protein regulation by monoubiquitin." Nat Rev Mol Cell Biol **2**(3): 195-201.

Jiang, Y. H., D. Armstrong, U. Albrecht, C. M. Atkins, J. L. Noebels, G. Eichele, J. D. Sweatt and A. L. Beaudet (1998). "Mutation of the Angelman ubiquitin ligase in mice causes increased cytoplasmic p53 and deficits of contextual learning and long-term potentiation." Neuron **21**(4): 799-811.

Kahl, P., L. Gullotti, L. C. Heukamp, S. Wolf, N. Friedrichs, R. Vorreuther, G. Solleder, P. J. Bastian, J. Ellinger, E. Metzger, R. Schule and R. Buettner (2006). "Androgen receptor coactivators lysine-specific histone demethylase 1 and four and a half LIM domain protein 2 predict risk of prostate cancer recurrence." Cancer Res **66**(23): 11341-11347.

Kaidanovich-Beilin, O., T. Lipina, I. Vukobradovic, J. Roder and J. R. Woodgett (2011). "Assessment of social interaction behaviors." J Vis Exp(48).

Kameyama, K., H. K. Lee, M. F. Bear and R. L. Huganir (1998). "Involvement of a postsynaptic protein kinase A substrate in the expression of homosynaptic long-term depression." Neuron **21**(5): 1163-1175.

Kato, A., N. Rouach, R. A. Nicoll and D. S. Bredt (2005). "Activity-dependent NMDA receptor degradation mediated by retrotranslocation and ubiquitination." Proc Natl Acad Sci U S A **102**(15): 5600-5605.

Khoriaty, R., G. G. Hesketh, A. Bernard, A. C. Weyand, D. Mellacheruvu, G. Zhu, M. J. Hoenerhoff, B. McGee, L. Everett, E. J. Adams, B. Zhang, T. L. Saunders, A. I. Nesvizhskii, D. J. Klionsky, J. A. Shavit, A. C. Gingras and D. Ginsburg (2018). "Functions of the COPII gene paralogs SEC23A and SEC23B are interchangeable in vivo." Proc Natl Acad Sci U S A **115**(33): E7748-E7757.

Kim, D., H. J. Nam, W. Lee, H. Y. Yim, J. Y. Ahn, S. W. Park, H. R. Shin, R. Yu, K. J. Won, J. S. Bae, K. I. Kim and S. H. Baek (2018). "PKCalpha-LSD1-NF-kappaB-Signaling Cascade Is Crucial for Epigenetic Control of the Inflammatory Response." Mol Cell **69**(3): 398-411 e396.

Kim, J. J. and M. W. Jung (2006). "Neural circuits and mechanisms involved in Pavlovian fear conditioning: a critical review." Neurosci Biobehav Rev **30**(2): 188-202.

Kim, S., T. Kim, H. R. Lee, Y. Y. Kong and B. K. Kaang (2015). "Mind Bomb-2 Regulates Hippocampus-dependent Memory Formation and Synaptic Plasticity." Korean J Physiol Pharmacol **19**(6): 515-522.

Kleiman, R. J., D. S. Chapin, C. Christoffersen, J. Freeman, K. R. Fonseca, K. F. Geoghegan, S. Grimwood, V. Guanowsky, M. Hajos, J. F. Harms, C. J. Helal, W. E. Hoffmann, G. P. Kocan, M. J. Majchrzak, D. McGinnis, S. McLean, F. S. Menniti, F. Nelson, R. Roof, A. W. Schmidt, P. A. Seymour, D. T. Stephenson, F. D. Tingley, M. Vanase-Frawley, P. R. Verhoest and C. J. Schmidt (2012). "Phosphodiesterase 9A regulates central cGMP and modulates responses to cholinergic and monoaminergic perturbation in vivo." J Pharmacol Exp Ther **341**(2): 396-409.

Koo, B. K., M. J. Yoon, K. J. Yoon, S. K. Im, Y. Y. Kim, C. H. Kim, P. G. Suh, Y. N. Jan and Y. Y. Kong (2007). "An obligatory role of mind bomb-1 in notch signaling of mammalian development." PLoS One **2**(11): e1221.

Koutelou, E., S. Sato, C. Tomomori-Sato, L. Florens, S. K. Swanson, M. P. Washburn, M. Kokkinaki, R. C. Conaway, J. W. Conaway and N. K. Moschonas (2008). "Neuralized-like 1 (Neurl1) targeted to the plasma membrane by N-myristoylation regulates the Notch ligand Jagged1." J Biol Chem **283**(7): 3846-3853.

Kubota, M., T. Murakoshi, H. Saegusa, A. Kazuno, S. Zong, Q. Hu, T. Noda and T. Tanabe (2001). "Intact LTP and fear memory but impaired spatial memory in mice

lacking Ca(v)2.3 (alpha(IE)) channel." Biochem Biophys Res Commun **282**(1): 242-248.

Kuleshov, M. V., M. R. Jones, A. D. Rouillard, N. F. Fernandez, Q. N. Duan, Z. C. Wang, S. Koplev, S. L. Jenkins, K. M. Jagodnik, A. Lachmann, M. G. McDermott, C. D. Monteiro, G. W. Gundersen and A. Ma'ayan (2016). "Enrichr: a comprehensive gene set enrichment analysis web server 2016 update." Nucleic Acids Research **44**(W1): W90-W97.

Lai, K. O., A. S. L. Wong, M. C. Cheung, P. Xu, Z. Y. Liang, K. C. Lok, H. Xie, M. E. Palko, W. H. Yung, L. Tessarollo, Z. H. Cheung and N. Y. Ip (2012). "TrkB phosphorylation by Cdk5 is required for activity-dependent structural plasticity and spatial memory." Nature Neuroscience **15**(11): 1506-1515.

Lee, H. K. (2006). "Synaptic plasticity and phosphorylation." Pharmacol Ther **112**(3): 810-832.

Lee, H. K., K. Takamiya, J. S. Han, H. Y. Man, C. H. Kim, G. Rumbaugh, S. Yu, L. Ding, C. He, R. S. Petralia, R. J. Wenthold, M. Gallagher and R. L. Huganir (2003). "Phosphorylation of the AMPA receptor GluR1 subunit is required for synaptic plasticity and retention of spatial memory." Cell **112**(5): 631-643.

Lee, K., Y. Kobayashi, H. Seo, J. H. Kwak, A. Masuda, C. S. Lim, H. R. Lee, S. J. Kang, P. Park, S. E. Sim, N. Kogo, H. Kawasaki, B. K. Kaang and S. Itohara (2015). "Involvement of cAMP-guanine nucleotide exchange factor II in hippocampal long-term depression and behavioral flexibility." Mol Brain **8**: 38.

Lehmann, R., F. Jimenez, U. Dietrich and J. A. Campos-Ortega (1983). "On the phenotype and development of mutants of early neurogenesis inDrosophila melanogaster." Wilehm Roux Arch Dev Biol **192**(2): 62-74.

Lim, S., A. Janzer, A. Becker, A. Zimmer, R. Schule, R. Buettner and J. Kirfel (2010). "Lysine-specific demethylase 1 (LSD1) is highly expressed in ER-negative breast cancers and a biomarker predicting aggressive biology." Carcinogenesis **31**(3): 512-520.

Lin, A. W. and H. Y. Man (2013). "Ubiquitination of neurotransmitter receptors and postsynaptic scaffolding proteins." Neural Plast **2013**: 432057.

Lussier, M. P., A. Sanz-Clemente and K. W. Roche (2015). "Dynamic Regulation of

N-Methyl-d-aspartate (NMDA) and alpha-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid (AMPA) Receptors by Posttranslational Modifications." J Biol Chem **290**(48): 28596-28603.

Mabb, A. M. and M. D. Ehlers (2010). "Ubiquitination in postsynaptic function and plasticity." Annu Rev Cell Dev Biol **26**: 179-210.

Malenka, R. C. and M. F. Bear (2004). "LTP and LTD: an embarrassment of riches." Neuron **44**(1): 5-21.

Malleret, G., R. Hen, J. L. Guillou, L. Segu and M. C. Buhot (1999). "5-HT1B receptor knock-out mice exhibit increased exploratory activity and enhanced spatial memory performance in the Morris water maze." J Neurosci **19**(14): 6157-6168.

Manning, G., D. B. Whyte, R. Martinez, T. Hunter and S. Sudarsanam (2002). "The protein kinase complement of the human genome." Science **298**(5600): 1912-1934.

Maren, S. and W. Holt (2000). "The hippocampus and contextual memory retrieval in Pavlovian conditioning." Behav Brain Res **110**(1-2): 97-108.

Matthies, H. and K. G. Reymann (1993). "Protein kinase A inhibitors prevent the maintenance of hippocampal long-term potentiation." Neuroreport **4**(6): 712-714.

Metzger, E., M. Wissmann, N. Yin, J. M. Muller, R. Schneider, A. H. Peters, T. Gunther, R. Buettner and R. Schule (2005). "LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription." Nature **437**(7057): 436-439.

Morris, R. G., P. Garrud, J. N. Rawlins and J. O'Keefe (1982). "Place navigation impaired in rats with hippocampal lesions." Nature **297**(5868): 681-683.

Mulkey, R. M., S. Endo, S. Shenolikar and R. C. Malenka (1994). "Involvement of a calcineurin/inhibitor-1 phosphatase cascade in hippocampal long-term depression." Nature **369**(6480): 486-488.

Mulkey, R. M., C. E. Herron and R. C. Malenka (1993). "An essential role for protein phosphatases in hippocampal long-term depression." Science **261**(5124): 1051-1055.

Mulligan, P., F. Yang, L. Di Stefano, J. Y. Ji, J. Ouyang, J. L. Nishikawa, D. Toiber, M. Kulkarni, Q. Wang, S. H. Najafi-Shoushtari, R. Mostoslavsky, S. P. Gygi, G. Gill, N. J. Dyson and A. M. Naar (2011). "A SIRT1-LSD1 corepressor complex regulates

Notch target gene expression and development." Mol Cell **42**(5): 689-699.

Murai, T., S. Okuda, T. Tanaka and H. Ohta (2007). "Characteristics of object location memory in mice: Behavioral and pharmacological studies." Physiol Behav **90**(1): 116-124.

Nakamura, H., M. Yoshida, H. Tsuiki, K. Ito, M. Ueno, M. Nakao, K. Oka, M. Tada, M. Kochi, J. Kuratsu, Y. Ushio and H. Saya (1998). "Identification of a human homolog of the Drosophila neuralized gene within the 10q25.1 malignant astrocytoma deletion region." Oncogene **16**(8): 1009-1019.

Nam, H. J., K. Boo, D. Kim, D. H. Han, H. K. Choe, C. R. Kim, W. Sun, H. Kim, K. Kim, H. Lee, E. Metzger, R. Schuele, S. H. Yoo, J. S. Takahashi, S. Cho, G. H. Son and S. H. Baek (2014). "Phosphorylation of LSD1 by PKCalpha is crucial for circadian rhythmicity and phase resetting." Mol Cell **53**(5): 791-805.

Nandi, D., P. Tahiliani, A. Kumar and D. Chandu (2006). "The ubiquitin-proteasome system." J Biosci **31**(1): 137-155.

Neelamegam, R., E. L. Ricq, M. Malvaez, D. Patnaik, S. Norton, S. M. Carlin, I. T. Hill, M. A. Wood, S. J. Haggarty and J. M. Hooker (2012). "Brain-penetrant LSD1 inhibitors can block memory consolidation." ACS Chem Neurosci **3**(2): 120-128.

Nicoll, R. A. (2017). "A Brief History of Long-Term Potentiation." Neuron **93**(2): 281-290.

Noree, C., N. Sirinonthanawech and J. E. Wilhelm (2019). "Saccharomyces cerevisiae ASN1 and ASN2 are asparagine synthetase paralogs that have diverged in their ability to polymerize in response to nutrient stress." Scientific Reports **9**.

Nussinov, R., C. J. Tsai, F. Xin and P. Radivojac (2012). "Allosteric post-translational modification codes." Trends Biochem Sci **37**(10): 447-455.

O'Callaghan, P., Y. Zarb, F. Noborn and J. Kreuger (2018). "Modeling the structural implications of an alternatively spliced Exoc3l2, a paralog of the tunneling nanotube-forming M-Sec." PLoS One **13**(8): e0201557.

O'Keefe, J. and N. Burgess (1996). "Geometric determinants of the place fields of hippocampal neurons." Nature **381**(6581): 425-428.

O'Keefe, J. and L. Nadel (1978). The hippocampus as a cognitive map. Oxford New York, Clarendon Press ; Oxford University Press.

Park, H., J. Yang, R. Kim, Y. Li, Y. Lee, C. Lee, J. Park, D. Lee, H. Kim and E. Kim (2015). "Mice lacking the PSD-95-interacting E3 ligase, Dorfin/Rnf19a, display reduced adult neurogenesis, enhanced long-term potentiation, and impaired contextual fear conditioning." Sci Rep **5**: 16410.

Pasinelli, P., G. M. Ramakers, I. J. Urban, J. J. Hens, A. B. Oestreicher, P. N. de Graan and W. H. Gispen (1995). "Long-term potentiation and synaptic protein phosphorylation." Behav Brain Res **66**(1-2): 53-59.

Pavlopoulos, E., M. Anezaki and E. M. Skoulakis (2008). "Neuralized is expressed in the alpha/beta lobes of adult Drosophila mushroom bodies and facilitates olfactory long-term memory formation." Proc Natl Acad Sci U S A **105**(38): 14674-14679.

Pavlopoulos, E., M. Kokkinaki, E. Koutelou, T. A. Mitsiadis, P. Prinos, C. Delidakis, M. W. Kilpatrick, P. Tsipouras and N. K. Moschonas (2002). "Cloning, chromosomal organization and expression analysis of Neurl, the mouse homolog of Drosophila melanogaster neuralized gene." Biochim Biophys Acta **1574**(3): 375-382.

Pavlopoulos, E., P. Trifilieff, V. Chevaleyre, L. Fioriti, S. Zairis, A. Pagano, G. Malleret and E. R. Kandel (2011). "Neuralized1 activates CPEB3: a function for nonproteolytic ubiquitin in synaptic plasticity and memory storage." Cell **147**(6): 1369-1383.

Paylor, R., M. Nguyen, J. N. Crawley, J. Patrick, A. Beaudet and A. Orr-Urtreger (1998). "alpha 7 nicotinic receptor subunits are not necessary for hippocampal-dependent learning or sensorimotor gating: A behavioral characterization of Acra7-deficient mice." Learning & Memory **5**(4-5): 302-316.

Pedersen, M. T. and K. Helin (2010). "Histone demethylases in development and disease." Trends Cell Biol **20**(11): 662-671.

Pinne, M., K. Denker, E. Nilsson, R. Benz and S. Bergstrom (2006). "The BBA01 protein, a member of paralog family 48 from Borrelia burgdorferi, is potentially interchangeable with the channel-forming protein P13." Journal of Bacteriology **188**(12): 4207-4217.

Poulin, B., A. Butcher, P. McWilliams, J. M. Bourgognon, R. Pawlak, K. C. Kong, A. Bottrill, S. Mistry, J. Wess, E. M. Rosethorne, S. J. Charlton and A. B. Tobin (2010). "The M3-muscarinic receptor regulates learning and memory in a receptor

phosphorylation/arrestin-dependent manner." Proc Natl Acad Sci U S A **107**(20): 9440-9445.

Powell, C. M., S. Schoch, L. Monteggia, M. Barrot, M. F. Matos, N. Feldmann, T. C. Sudhof and E. J. Nestler (2004). "The presynaptic active zone protein RIM1 alpha is critical for normal learning and memory." Neuron **42**(1): 143-153.

Price, B. D., Z. Chang, R. Smith, S. Bockheim and A. Laughon (1993). "The Drosophila neuralized gene encodes a C3HC4 zinc finger." EMBO J **12**(6): 2411-2418.

Prut, L. and C. Belzung (2003). "The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review." Eur J Pharmacol **463**(1-3): 3-33.

Ramos, A. (2008). "Animal models of anxiety: do I need multiple tests?" Trends Pharmacol Sci **29**(10): 493-498.

Rochford, J., S. Beaulieu, I. Rousse, J. R. Glowa and N. Barden (1997). "Behavioral reactivity to aversive stimuli in a transgenic mouse model of impaired glucocorticoid (type II) receptor function: effects of diazepam and FG-7142." Psychopharmacology (Berl) **132**(2): 145-152.

Routtenberg, A. and J. L. Rekart (2005). "Post-translational protein modification as the substrate for long-lasting memory." Trends in Neurosciences **28**(1): 12-19.

Ruan, Y., L. Tecott, M. M. Jiang, L. Y. Jan and Y. N. Jan (2001). "Ethanol hypersensitivity and olfactory discrimination defect in mice lacking a homolog of Drosophila neuralized." Proc Natl Acad Sci U S A **98**(17): 9907-9912.

Rudolph, T., S. Beuch and G. Reuter (2013). "Lysine-specific histone demethylase LSD1 and the dynamic control of chromatin." Biol Chem **394**(8): 1019-1028.

Rullinkov, G., R. Tamme, A. Sarapuu, J. Lauren, M. Sepp, K. Palm and T. Timmusk (2009). "Neuralized-2: expression in human and rodents and interaction with Delta-like ligands." Biochem Biophys Res Commun **389**(3): 420-425.

Sacktor, T. C. (2008). "PKM zeta, LTP maintenance, and the dynamic molecular biology of memory storage." Essence of Memory **169**: 27-40.

Sacktor, T. C., P. Osten, H. Valsamis, X. Jiang, M. U. Naik and E. Sublette (1993). "Persistent activation of the zeta isoform of protein kinase C in the maintenance of long-term potentiation." Proc Natl Acad Sci U S A **90**(18): 8342-8346.

Santini, E., T. N. Huynh and E. Klann (2014). "Mechanisms of translation control underlying long-lasting synaptic plasticity and the consolidation of long-term memory." Prog Mol Biol Transl Sci **122**: 131-167.

Schoch, S., P. E. Castillo, T. Jo, K. Mukherjee, M. Geppert, Y. Wang, F. Schmitz, R. C. Malenka and T. C. Sudhof (2002). "RIM1 alpha forms a protein scaffold for regulating neurotransmitter release at the active zone." Nature **415**(6869): 321-326.

Schreiber, J., M. J. Vegh, J. Dawitz, T. Kroon, M. Loos, D. Labonte, K. W. Li, P. Van Nierop, M. T. Van Diepen, C. I. De Zeeuw, M. Kneussel, R. M. Meredith, A. B. Smit and R. E. Van Kesteren (2015). "Ubiquitin ligase TRIM3 controls hippocampal plasticity and learning by regulating synaptic gamma-actin levels." J Cell Biol **211**(3): 569-586.

Serrano, P., Y. Yao and T. C. Sacktor (2005). "Persistent phosphorylation by protein kinase Mzeta maintains late-phase long-term potentiation." J Neurosci **25**(8): 1979-1984.

Shepherd, J. K., S. S. Grewal, A. Fletcher, D. J. Bill and C. T. Dourish (1994). "Behavioural and pharmacological characterisation of the elevated "zero-maze" as an animal model of anxiety." Psychopharmacology (Berl) **116**(1): 56-64.

Shi, Y., F. Lan, C. Matson, P. Mulligan, J. R. Whetstine, P. A. Cole, R. A. Casero and Y. Shi (2004). "Histone demethylation mediated by the nuclear amine oxidase homolog LSD1." Cell **119**(7): 941-953.

Silva, A. J., T. W. Rosahl, P. F. Chapman, Z. Marowitz, E. Friedman, P. W. Frankland, V. Cestari, D. Cioffi, T. C. Sudhof and R. Bourtchuladze (1996). "Impaired learning in mice with abnormal short-lived plasticity." Curr Biol **6**(11): 1509-1518.

Silva, A. J., C. F. Stevens, S. Tonegawa and Y. Wang (1992). "Deficient hippocampal long-term potentiation in alpha-calcium-calmodulin kinase II mutant mice." Science **257**(5067): 201-206.

Snider, K. H., H. Dziema, S. Aten, J. Loeser, F. E. Norona, K. Hoyt and K. Obrietan (2016). "Modulation of learning and memory by the targeted deletion of the circadian clock gene Bmal1 in forebrain circuits." Behav Brain Res **308**: 222-235.

Song, L. and Z. Q. Luo (2019). "Post-translational regulation of ubiquitin signaling." J Cell Biol **218**(6): 1776-1786.

Song, R., B. K. Koo, K. J. Yoon, M. J. Yoon, K. W. Yoo, H. T. Kim, H. J. Oh, Y. Y. Kim, J. K. Han, C. H. Kim and Y. Y. Kong (2006). "Neuralized-2 regulates a Notch ligand in cooperation with Mind bomb-1." J Biol Chem **281**(47): 36391-36400.

Strohle, A., M. Poettig, N. Barden, F. Holsboer and A. Montkowski (1998). "Age- and stimulus-dependent changes in anxiety-related behaviour of transgenic mice with GR dysfunction." Neuroreport **9**(9): 2099-2102.

Sun, J., G. Zhu, Y. Liu, S. Standley, A. Ji, R. Tunuguntla, Y. Wang, C. Claus, Y. Luo, M. Baudry and X. Bi (2015). "UBE3A Regulates Synaptic Plasticity and Learning and Memory by Controlling SK2 Channel Endocytosis." Cell Rep **12**(3): 449-461.

Sunyer, B., W. Diao and G. Lubec (2008). "The role of post-translational modifications for learning and memory formation." Electrophoresis **29**(12): 2593-2602.

Taal, K., J. Tuvikene, G. Rullinkov, M. Piirsoo, M. Sepp, T. Neuman, R. Tamme and T. Timmusk (2019). "Neuralized family member NEURL1 is a ubiquitin ligase for the cGMP-specific phosphodiesterase 9A." Sci Rep **9**(1): 7104.

Takagi, H., M. Setou, S. Ito and I. Yao (2012). "SCRAPPER regulates the thresholds of long-term potentiation/depression, the bidirectional synaptic plasticity in hippocampal CA3-CA1 synapses." Neural Plast **2012**: 352829.

Timmusk, T., K. Palm, N. Belluardo, G. Mudo and T. Neuman (2002). "Dendritic localization of mammalian neuralized mRNA encoding a protein with transcription repression activities." Mol Cell Neurosci **20**(4): 649-668.

Tsokas, P., C. Hsieh, Y. Yao, E. Lesburgueres, E. J. C. Wallace, A. Tcherepanov, D. Jothianandan, B. R. Hartley, L. Pan, B. Rivard, R. V. Farese, M. P. Sajan, P. J. Bergold, A. I. Hernandez, J. E. Cottrell, H. Z. Shouval, A. A. Fenton and T. C. Sacktor (2016). "Compensation for PKMzeta in long-term potentiation and spatial long-term memory in mutant mice." Elife **5**.

Tu, M., P. Zhu, S. Hu, W. Wang, Z. Su, J. Guan, C. Sun and W. Zheng (2017). "Notch1 Signaling Activation Contributes to Adult Hippocampal Neurogenesis Following Traumatic Brain Injury." Med Sci Monit **23**: 5480-5487.

van der Staay, F. J., K. Rutten, L. Barfacker, J. Devry, C. Erb, H. Heckroth, D. Karthaus, A. Tersteegen, M. van Kampen, A. Blokland, J. Prickaerts, K. G.

Reymann, U. H. Schroder and M. Hendrix (2008). "The novel selective PDE9 inhibitor BAY 73-6691 improves learning and memory in rodents." Neuropharmacology **55**(5): 908-918.

Vogel-Ciernia, A. and M. A. Wood (2014). "Examining object location and object recognition memory in mice." Curr Protoc Neurosci **69**: 8 31 31-17.

Vorhees, C. V. and M. T. Williams (2006). "Morris water maze: procedures for assessing spatial and related forms of learning and memory." Nature Protocols **1**(2): 848-858.

Walsh, C. (2006). Posttranslational modification of proteins : expanding nature's inventory. Englewood, Colo., Roberts and Co. Publishers.

Wang, J., S. Hevi, J. K. Kurash, H. Lei, F. Gay, J. Bajko, H. Su, W. Sun, H. Chang, G. Xu, F. Gaudet, E. Li and T. Chen (2009). "The lysine demethylase LSD1 (KDM1) is required for maintenance of global DNA methylation." Nat Genet **41**(1): 125-129.

Wang, J., F. Telese, Y. Tan, W. Li, C. Jin, X. He, H. Basnet, Q. Ma, D. Merkurjev, X. Zhu, Z. Liu, J. Zhang, K. Ohgi, H. Taylor, R. R. White, C. Tazearslan, Y. Suh, T. S. Macfarlan, S. L. Pfaff and M. G. Rosenfeld (2015). "LSD1n is an H4K20 demethylase regulating memory formation via transcriptional elongation control." Nat Neurosci **18**(9): 1256-1264.

Wang, Y., S. L. Chan, L. Miele, P. J. Yao, J. Mackes, D. K. Ingram, M. P. Mattson and K. Furukawa (2004). "Involvement of Notch signaling in hippocampal synaptic plasticity." Proc Natl Acad Sci U S A **101**(25): 9458-9462.

Wardlaw, S. M., T. X. Phan, A. Saraf, X. Chen and D. R. Storm (2014). "Genetic disruption of the core circadian clock impairs hippocampus-dependent memory." Learn Mem **21**(8): 417-423.

Watabe, A. M., M. Nagase, A. Hagiwara, Y. Hida, M. Tsuji, T. Ochiai, F. Kato and T. Ohtsuka (2016). "SAD-B kinase regulates pre-synaptic vesicular dynamics at hippocampal Schaffer collateral synapses and affects contextual fear memory." Journal of Neurochemistry **136**(1): 36-47.

Wehner, J. M. and R. A. Radcliffe (2004). "Cued and contextual fear conditioning in mice." Curr Protoc Neurosci **Chapter 8**: Unit 8 5C.

Widagdo, J., S. Guntupalli, S. E. Jang and V. Anggono (2017). "Regulation of AMPA

Receptor Trafficking by Protein Ubiquitination." Front Mol Neurosci **10**: 347.

Wilkinson, K. D. (1987). "Protein ubiquitination: a regulatory post-translational modification." Anticancer Drug Des **2**(2): 211-229.

Yang, M., C. B. Gocke, X. Luo, D. Borek, D. R. Tomchick, M. Machius, Z. Otwinowski and H. Yu (2006). "Structural basis for CoREST-dependent demethylation of nucleosomes by the human LSD1 histone demethylase." Mol Cell **23**(3): 377-387.

Yeh, E., M. Dermer, C. Commisso, L. Zhou, C. J. McGlade and G. L. Boulianne (2001). "Neuralized functions as an E3 ubiquitin ligase during Drosophila development." Curr Biol **11**(21): 1675-1679.

Zhang, Q., Y. Li, L. Zhang, N. Yang, J. Meng, P. Zuo, Y. Zhang, J. Chen, L. Wang, X. Gao and D. Zhu (2013). "E3 ubiquitin ligase RNF13 involves spatial learning and assembly of the SNARE complex." Cell Mol Life Sci **70**(1): 153-165.

Zheng, N. and N. Shabek (2017). "Ubiquitin Ligases: Structure, Function, and Regulation." Annu Rev Biochem **86**: 129-157.

Zibetti, C., A. Adamo, C. Binda, F. Forneris, E. Toffolo, C. Verpelli, E. Ginelli, A. Mattevi, C. Sala and E. Battaglioli (2010). "Alternative splicing of the histone demethylase LSD1/KDM1 contributes to the modulation of neurite morphogenesis in the mammalian nervous system." J Neurosci **30**(7): 2521-2532.

Zucker, R. S. and W. G. Regehr (2002). "Short-term synaptic plasticity." Annu Rev Physiol **64**: 355-405.

국문초록

학습과 기억은 수많은 분자적 기전들을 통해 조절된다. 그동안 많은 연구들을 통해 단백질의 번역 후 변형(Post-translational modification)이 학습과 기억에 중요한 역할을 한다는 점이 알려졌지만 여전히 밝혀내야 할 부분이 많이 남아있다. 나는 이 연구에서 여러 종류의 번역 후 변형 메커니즘 중에서 특히 단백질의 인산화 (Phosphorylation)와 유비퀴틴화 (Ubiquitination)가 학습과 기억에 미치는 영향에 대해서 연구하고자 하였다. 이를 위해 형질전환 생쥐 모델을 이용하는 일련의 실험을 진행하였으며 이 두 종류의 번역 후 변형 메커니즘이 학습과 기억에 관여한다는 생물학적 증거를 제시하였다.

첫 번째 연구에서 나는 단백질 인산화효소 Cα (PKCα)로 매개되는 Lysine-specific demethylase 1 (Lsd1) 단백질의 인산화가 해마 의존적 학습과 기억에 미치는 영향을 알아보기 위해서 단백질 인산화효소 Cα로 매개되는 인산화가 결핍된 Lsd1 유전자를 삽입한 생쥐 (*Lsd1* KI 생쥐)를 이용하였다. 행동 실험을 진행한 결과 *Lsd1* KI 생쥐는 대조군 (WT littermate)에 비해서 해마 의존적 공포 기억과 공간 기억이 저해된 것을 관찰할 수 있었다. 또한, 전기생리학 실험을 통해서 *Lsd1* KI 생쥐의 시냅스전 말단의 기능이 향상되어 있는 것을 관찰 할 수 있었는데, 이는 단기 시냅스 가소성 (Short-term synaptic plasticity)과 관련이 있다. 한편, 장기강화 (Long-term potentiation: LTP)와 장기억압 (Long-term

depression: LTD)과 같은 장기 시냅스 가소성 (Long-term synaptic plasticity)은 *Lsd1* KI 생쥐에게서 정상적으로 보존되어 있는 것이 관찰되였다. 뿐만 아니라, *Lsd1* KI 생쥐의 해마 조직에 대해 RNA-seq 분석을 진행한 결과에서도 시냅스 전 말단의 기능과 관련된 유전자 발현량의 증가를 확인할 수 있었다. 본 연구는 위 결과들로부터 단백질 인산화효소 C α (PKC α)로 매개되는 Lsd1 단백질 인산화가 생리적, 분자적 수준에서 시냅스전 말단의 기능을 조절하며 학습과 기억에 영향을 미친다는 해석을 이끌어내었다.

두 번째 연구에서 나는 E3 유비퀴틴 연결효소 (E3 ubiquition ligase)인 Neurl 1 과 Neurl 2 가 해마 의존적 학습과 기억에 미치는 영향에 대해서 알아보았다. 이를 위해서 *Neurl 1* 유전자가 결손된 *Neurl 1* 녹아웃 생쥐 (*Neurl 1* KO 생쥐)와 *Neurl 2* 유전자가 결손된 *Neurl 2* 녹아웃 생쥐 (*Neurl 2* KO 생쥐), 그리고 *Neurl 1*과 *Neurl 2* 유전자가 모두 결손된 *Neurl 1, 2* 녹아웃 생쥐 (*Neurl 1,2* KO 생쥐)를 이용하였다. 행동 실험을 진행한 결과, 대조군 생쥐에 비해서 *Neurl 1,2* KO 생쥐에서만 해마 의존적 공간 기억이 저해된 점을 확인하였다. 또한, 전기생리학 실험을 통해서 *Neurl 1* KO 생쥐, *Neurl 2* KO 생쥐, *Neurl 1,2* KO 생쥐 모두 시냅스 기저 특성 (Basal synaptic property) 및 초기 장기강화 (Earl-phase LTP)에는 변화가 없었던 반면 *Neurl 1,2* KO 생쥐에서만 단백질 합성 의존적 장기 강화 (Protein synthesis-dependent LTP)가 저해되어 있는 사실을 확인할 수 있었다. 마지막으로 *Neurl 1* 과 *Neurl 2*이 서로에 대해서

상보적인 역할을 수행할 가능성을 시험해 보기 위해서 Quantitive-real time PCR 실험을 진행하였으며, *Neurl 1* KO 생쥐에서는 *Neurl 2* 의 전사물의, 그리고 *Neurl 2* KO 생쥐에서는 *Neurl 1* 의 전사물의 양이 증가해 있지 않다는 결과를 얻었다. 이러한 결과들을 종합해 보면, *Neurl 1* 혹은 *Neurl 2* 중 적어도 하나의 유전자가 있는 상황에서 해마 의존적 공간 기억과 단백질 합성 의존적 장기 강화는 손상되지 않고 유지되지만, *Neurl 1* 과 *Neurl 2* 전사물의 양을 비교해 보았을 때 이 두 가지 유전자가 그 과정에서 서로 상보적인 역할을 하지는 않는다는 결론을 내릴 수 있다.

요약하자면 나는 번역 후 변형에 관련된 두 가지 연구로서, 각각 단백질 인산화효소 Cα (PKCα)로 매개되는 Lsd1 단백질의 인산화와 E3 유비퀴틴 연결 효소인 Neurl 1 과 Neurl 2 가 해마 의존적 학습과 기억을 조절한다는 것을 밝혀냈다. 한편, 이 두 연구는 서로 다른 생리학적 특성과 분자적 기전을 통해서 해마 의존적 기억이 조절된다는 것을 보여주었다는 점에서 번역 후 변형이 다양한 분자적 기전을 통해 학습과 기억을 조절할 수 있다는 견해를 지지한다.

주요어: 인산화, 유비퀴틴화, 시냅스 가소성, 히스톤 탈메틸화효소, E3 유비퀴틴 연결 효소, 해마

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